

WEST Search History

DATE: Tuesday, December 09, 2003

Set Name Query

side by side

Hit Count Set Name

result set

DB=USPT; PLUR=YES; OP=AND

L1	43504	105	L1
L2	L1 and pylori	49	L2
L3	L1 and helicobacter	49	L3
L4	pronovost.in. and helicobacter	3	L4
L5	((43504) and helicobacter)	49	L5

END OF SEARCH HISTORY

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DATE: Tuesday, December 09, 2003

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DB=USPT; PLUR=YES; OP=AND

L1 eradica\$

L2 L1 same (pylori or pylori or pyloridis or
pylorum or hpylori or helicobacter)

L3 before same during same after

L4 L3 and l2

L5 L4 and titer

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result set

6332 L1

212 L2

111823 L3

20 L4

4 L5

END OF SEARCH HISTORY

WEST Search History

DATE: Tuesday, December 09, 2003

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result set

DB=USPT; PLUR=YES; OP=AND

L1	eradica\$	6332	L1
L2	L1 same (pylori or pyloris or pyloridis or pylorum or hpylori or helicobacter)	212	L2
L3	before same during same after	111823	L3
L4	L3 and l2	20	L4
L5	L4 and titer	4	L5
	(before or prior or diagnose or diagnostic)		
L6	same (during or monoitor or monitoring) same (post or after or eradication)	179598	L6
L7	L6 same (pylori or pyloris or pyloridis or pylorum or hpylori or helicobacter)	24	L7
	L7 same (antihuman or anti-human or anti-h		
L8	or antih or antihelicobacter or titer or antibodies or elisa or eia or blotting or western or immunoblotting or immunoblot)	8	L8

END OF SEARCH HISTORY

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- ☐ 1. 6303125. 24 Sep 98; 16 Oct 01. Anti-microbial-adhesion fraction derived from vaccinium. Ofek; Itzhak, et al. 424/732; 424/405 424/408 424/410 424/417 424/440 514/783. A01N065/00.
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- ☐ 4. 5837240. 26 Aug 97; 17 Nov 98. Multimeric, recombinant urease vaccine. Lee; Cynthia K., et al. 424/94.6; 424/234.1 435/227 514/925 514/926 514/927. A61K038/46 A61K039/02 A61K039/106.
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Terms	Documents
L4 and titer	4

[Previous Page](#)[Next Page](#)

09842863 21654164 PMID: 11795863

Helicobacter pylori (H. pylori) infection in coronary artery disease: influence of H. pylori eradication on coronary artery lumen after percutaneous transluminal coronary angioplasty. The detection of H. pylori specific DNA in human coronary atherosclerotic plaque.

Kowalski M

Department of Cardiology, Heart-Center Osnabruck-Bad Rothenfelde, Germany.

Journal of physiology and pharmacology - an official journal of the Polish Physiological Society (Poland) Aug 2001, 52 (1 Suppl 1) p3-31, ISSN 0867-5910 Journal Code: 9114501

Document type: Clinical Trial; Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

BACKGROUND: The role of infections in pathogenesis of atherosclerosis has been a point of extensive discussion and research. Chronic infection has been proposed to account for the formation and progression of atherosclerotic plaques. Gastric mucosal damage caused by *Helicobacter pylori* (*H. pylori*) involves various bacterial and host-dependent toxic substances that have been recently associated with increased risk of coronary artery disease (CAD). **AIMS:** This study was designed to: 1) to determine the seroprevalence of *H. pylori* and its cytotoxin associated gene A (CagA) in patients with and without CAD (group A), 2) to evaluate the influence of infection with *H. pylori* expressing CagA (Cytotoxin associated gene A--as a determinant of high virulence) on coronary arterial lumen reduction in patients **after** percutaneous transluminal coronary angioplasty (PTCA) with stent (group B), 3) to assess the effect of *H. pylori* eradication on coronary artery lumen reduction **after** PTCA (group C), 4) to determine the influence of the *H. pylori* eradication on plasma levels of cytokines, lipids and coagulation factors in the same patients **before** and **after** PTCA (group C) and 5) to analyse coronary specimens in patients with severe CAD for the presence of *H. pylori* originated specific DNA (Group D). **PATIENTS AND METHODS:** Group A included 96 patients with CAD (subgroup I) and 96 patients without CAD (subgroup II). For *H. pylori* seroprevalence, plasma anti-*H. pylori* and anti-CagA **IgG** were examined by **ELISA** and **Western blot**. Group B included 135 patients (86 male, 49 female, mean age 67 +/- 12 years) who had underwent PTCA with stent implantation initially and recoronaro-angiography about 6 months **afterwards**. All patients were tested for *H. pylori*--specific **antibodies** (**IgG** and CagA). Patients were divided into three subgroup "a": 34 patients with *H. pylori* **IgG** and CagA seropositivity, subgroup "b": 37 patients infected with *H. pylori* positive and CagA negative germs and subgroup "c": 64 patients with *H. pylori* **IgG** sero-negativity serving as control group. For all patients coronary lumen loss (percentage) in the dilated segment was measured at the end of PTCA and **during** re-coronaro-angiography and obtained values were considered taking into account the major risk factors of CAD (hypertension, hyperlipidemia, diabetes, obesity and smoking). Group C included 40 patients with significant single-vessel CAD and *H. pylori* infection confirmed by 13C-urea breath test (UBT) and serologically using anti-*H. pylori* and anti-CagA **IgG**. In addition, plasma interleukin (IL)-1beta and IL-8 and tumor necrosis factor alpha (TNFalpha) levels were measured by **ELISA**. Plasma total triglycerides, cholesterol, low (LDL) and high density lipoproteins (HDL), homocysteine levels, as well as some clotting factors such as thromboplastin and fibrinogen levels, thrombin time and platelet count were determined. All patients of group B undergoing PTCA were divided into two matched subgroups I and II used in exploratory study; subgroup I (20 patients) received *H. pylori* eradication triple-therapy for one week (Clarithromycin, Amoxicillin and Omeprazole), while subgroup II (20 patients) received similarly prepared placebo for the same time period starting immediately **after** PTCA. Six months **after** PTCA, the *H. pylori* status was re-assessed by UBT and found to be negative in all but two patients of subgroup I subjected to *H. pylori* therapy. Coronary angiography and laboratory tests were repeated in both subgroups of group B included into the trial and the reduction in coronary artery lumen in these subgroups was compared to baseline **after** PTCA considered as 100%. Large atherosclerotic plaques from coronary endatherectomy were obtained in 46

consecutive patients (9 female, 37 male, mean age 63 +/- 9 years) during coronary bypass procedures (group D). Serum was analysed for positive **IgG antibodies** specific for *H. pylori* by enzyme-linked-immunosorbent assay (**ELISA**). **Antibodies** specific for the CagA were detected by immunoblot analysis. Polymerase chain reaction (PCR) was used to identify bacterial DNA with primers encoding for the 16 S ribosomal RNA of *H. pylori*. Sequence analysis of PCR-products confirmed the specificity of the gene products for *H. pylori*. Coronary artery biopsies from 19 autopsies from a Forensic Medicine Department without coronary atherosclerosis were examined as a control group. RESULTS: The *H. pylori* seropositivity reached 69.79% (67 pts) of CAD (subgroup I of group A) and it was significantly higher than that in controls without CAD (subgroup II)--40.62% (39 pts), the odds ratio (OR) being 3.38 95% CI: 1.8598-6.1306 for *H. pylori* in CAD. CagA **IgG** detection was also significantly higher (58.20%)

Tags: Female; Human; Male

Descriptors: *Arteriosclerosis--microbiology--MI; *Coronary Arteriosclerosis--complications--CO; *DNA, Bacterial--analysis--AN; *Helicobacter Infections--complications--CO; *Helicobacter pylori; Aged; Angioplasty, Transluminal, Percutaneous Coronary; **Antibodies**, Bacterial--analysis--AN; Arteriosclerosis--pathology--PA; Blood Coagulation Factors--metabolism--ME; Blotting, **Western**; Coronary Arteriosclerosis--microbiology--MI; Cytokines--blood--BL; Enzyme-Linked Immunosorbent Assay; Helicobacter Infections--drug therapy--DT; Helicobacter Infections--microbiology--MI; Helicobacter pylori--genetics--GE; Lipids--blood--BL; Middle Age; Risk Factors; Seroepidemiologic Studies

CAS Registry No.: 0 (Antibodies, Bacterial); 0 (Blood Coagulation Factors); 0 (Cytokines); 0 (DNA, Bacterial); 0 (Lipids)

Record Date Created: 20020117

Record Date Completed: 20020611

09780203 21586040 PMID: 11728547

Eradication of *Helicobacter pylori* and improvement of hereditary angioneurotic oedema.

Farkas H; Fust G; Fekete B; Karadi I; Varga L

Lancet (England) Nov 17 2001, 358 (9294) p1695-6, ISSN 0140-6736

Journal Code: 2985213R

Document type: Letter

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: AIM; INDEX MEDICUS

Helicobacter pylori infection is thought to be a causal factor in various dermatological disorders. We assessed the frequency of *H pylori* infection in 65 patients with hereditary angioneurotic oedema. We measured the serum concentration of **antibodies** against *H pylori* and did the carbon-14-urease breath test in patients with positive *H pylori* serology. 19 of 65 patients had *H pylori* infection. All patients with infection, and 11 of 46 without infection, had a history of recurrent episodes of acute abdominal pain. We successfully eradicated *H. pylori* infection in 18 patients. The frequency of abdominal symptoms was significantly higher in the infected group ($p=0.002$ **after** adjustment for age). In nine of 19 patients with dyspepsia, the frequency of oedematous episodes decreased from 100 over 10 months **before** eradication to 19 **during** the 10-month follow-up period. Screening for, and eradication of, *H pylori* infection seems to be justified in patients with hereditary angioneurotic oedema.

Tags: Female; Human; Male

Descriptors: *Abdominal Pain--etiology--ET; *Angioneurotic Edema --genetics--GE; *Complement 1 Inactivators--deficiency--DF; **Helicobacter* Infections--complications--CO; **Helicobacter pylori*; Adult; Angioneurotic Edema--microbiology--MI; **Antibodies**, Bacterial--blood--BL; Danazol --therapeutic use--TU; Estrogen Antagonists--therapeutic use--TU; *Helicobacter* Infections--drug therapy--DT

CAS Registry No.: 0 (Antibodies, Bacterial); 0 (Complement 1 Inactivators); 0 (Estrogen Antagonists); 17230-88-5 (Danazol)

Record Date Created: 20011130

Record Date Completed: 20011213

09687575 21479575 PMID: 11595060

An evaluation of the PyloriTek test for the diagnosis of Helicobacter pylori infection in Chinese patients before and after eradication therapy.

Wong W M; Wong B C; Tang V S; Lai K C; Yuen S T; Leung S Y; Hu W H; Lam S K

Department of Medicine, University of Hong Kong, Queen Mary Hospital, Pokfulam, Hong Kong.

Journal of gastroenterology and hepatology (Australia) Sep 2001, 16 (9) p976-80, ISSN 0815-9319 Journal Code: 8607909

Document type: Evaluation Studies; Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: Completed

Subfile: INDEX MEDICUS

BACKGROUND AND AIM: The PyloriTek Test Kit (a 1-h rapid urease test) was developed for the rapid diagnosis of Helicobacter pylori (H. pylori) **during** endoscopy. Most studies were performed in **Western** populations. The aim of this study was to evaluate the PyloriTek test for the diagnosis of H. pylori infection in Chinese population. **METHODS:** Eligible patients without prior treatment or who had had recent eradication of H. pylori were recruited. **During** endoscopy, biopsies were taken from the antrum and corpus for an in-house rapid urease test (RUT), histology and for the PyloriTek test (one antral and one corpus biopsy). Results of the PyloriTek test were compared with the gold standard (RUT and histology). **RESULTS:** Analysis of PyloriTek test results from the antrum alone (101 patients **before** eradication and 52 patients **after** eradication) showed a sensitivity, specificity, and accuracy of 96.3, 97.9, and 97.0%, respectively, for cases **before** eradication, and an accuracy of 100% for cases **after** eradication. The benefit of an additional body biopsy was marginal and only occurred in the pre-eradication group. **CONCLUSION:** The PyloriTek test was highly accurate for the diagnosis of H. pylori infection **before** and **after** eradication therapy, with a final result available at 1 h, which is unmatched by any invasive test so far. It enhances clinical decision-making by allowing the clinicians or endoscopists to start therapy on the same day of an endoscopy visit. One biopsy from the antrum is highly reliable for this purpose.

Tags: Human; Support, Non-U.S. Gov't

Descriptors: *Endoscopy, Digestive System; *Gastritis--diagnosis--DI; *Helicobacter Infections--diagnosis--DI; *Helicobacter pylori; *Peptic Ulcer--diagnosis--DI; *Reagent Strips; *Urease--analysis--AN; Anti-Ulcer Agents--therapeutic use--TU; Antibiotics--therapeutic use--TU; Biopsy; China; Gastric Mucosa--pathology--PA; Gastritis--drug therapy--DT; Gastritis--pathology--PA; Helicobacter Infections--drug therapy--DT; Helicobacter Infections--pathology--PA; Peptic Ulcer--drug therapy--DT; Peptic Ulcer--pathology--PA; Predictive Value of Tests; Prospective Studies

CAS Registry No.: 0 (Anti-Ulcer Agents); 0 (Antibiotics); 0 (Reagent Strips)

Enzyme No.: EC 3.5.1.5 (Urease)

Record Date Created: 20011011

Record Date Completed: 20011231

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File 155:MEDLINE(R) 1966-2003/Nov W4
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*File 155: Medline has temporarily stopped updating with
Completed records (Nov 2003). Please see HELP NEWS 154 for details.
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*File 159: Cancerlit ceases updating with immediate effect.
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*File 162: Effective May 1, name changes from CAB Health
to Global Health.
File 164:Allied & Complementary Medicine 1984-2003/Nov
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S2	10969	HELICOBACTER?/TI AND ERADICAT?/TI
S3	56	S2 AND (BEFORE? AND DURING? AND AFTER?) AND (IG OR IGG OR - IGA OR IMMUNE OR IMMUNOASSAY? OR ELISA? OR WESTERN? OR ELIZA? OR ANTIBOD?)

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3/9/11 (Item 5 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
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0012620602 BIOSIS NO.: 200000338915
Mucosal change of the stomach with low-grade mucosa-associated lymphoid tissue lymphoma after eradication of Helicobacter pylori: Follow-up study of 48 cases
AUTHOR: Begum Shahnaz; Sano Toshiaki (Reprint); Endo Hideko; Kawamata Hitoshi; Urakami Yoshihito
AUTHOR ADDRESS: Department of Pathology, University of Tokushima School of Medicine, Kuramoto-cho, Tokushima, 770-8503, Japan**Japan
JOURNAL: Journal of Medical Investigation 47 (1-2): p36-46 February, 2000
2000
MEDIUM: print
ISSN: 1343-1420
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Low-grade mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach has been demonstrated to be closely linked to Helicobacter pylori (H. pylori) and to be frequently remissioned **after** the cure of H. pylori infection. Several previous studies have focused on proliferating lymphocytes but little is known about gastric epithelial change and the duration of the remission **after** the cure of H. pylori infection. We performed a long-term follow-up investigation on the effects of anti-H. pylori treatment on MALT lymphoma and chronic gastritis at the histologic and molecular levels. Forty-eight patients with low-grade gastric MALT lymphoma and 28 chronic gastritis patients in whom H. pylori infection was eradicated were studied. **After** eradication, 43 MALT lymphoma patients showed complete histologic remission and continuous remission was observed **during** follow-up for up to 43 months (mean, 17.8 months). As for epithelial changes **after** eradication, "emptiness of lamina propria" was more pronounced in the mucosa with MALT lymphoma than that with chronic gastritis, and its severity in MALT lymphoma cases significantly decreased **during** the observation period whereas the glandular area increased. Cystic change of the fundic gland also occurred more frequently in MALT lymphoma cases than chronic gastritis cases. B-cell clonality **before** eradication analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) was detected in almost all MALT lymphoma cases (43 cases), but rare in chronic gastritis cases (6 cases). **After** eradication, in spite of histologic regression, 21 MALT lymphoma patients had a persistent monoclonal population **during** the follow-up period. B-cell monoclonality preceding the malignant transformation was noted in 4 cases. These observations indicate that 1) complete histologic remission of low-grade gastric MALT lymphomas seems stable even if a monoclonal B cell population is detectable in some cases, 2) there may be a stage of disease where monoclonal B cells are present but there is no histologic evidence of MALT lymphoma, and 3) regenerative change of the damaged glands may occur in histologic regressed MALT lymphoma cases.

DESCRIPTORS:

MAJOR CONCEPTS: Infection; Gastroenterology--Human Medicine, Medical Sciences; Hematology--Human Medicine, Medical Sciences; Oncology--Human Medicine, Medical Sciences
BIOSYSTEMATIC NAMES: Aerobic Helical or Vibrioid Gram-Negatives--

Eubacteria, Bacteria, Microorganisms; Hominidae--Primates, Mammalia, Vertebrata, Chordata, Animalia
 ORGANISMS: Helicobacter pylori (Aerobic Helical or Vibrioid Gram-Negatives)--pathogen; human (Hominidae)--patient
 ORGANISMS: PARTS ETC: B-cell--blood and lymphatics, **immune** system; gastric mucosa--digestive system; stomach--digestive system
 COMMON TAXONOMIC TERMS: Bacteria; Eubacteria; Microorganisms; Animals; Chordates; Humans; Mammals; Primates; Vertebrates
 DISEASES: Helicobacter pylori infection--bacterial disease, digestive system disease; chronic gastritis--digestive system disease; gastric mucosa-associated lymphoid tissue lymphoma {gastric MALT lymphoma}--blood and lymphatic disease, digestive system disease, **immune** system disease, neoplastic disease, low-grade
 MESH TERMS: Helicobacter Infections (MeSH); Gastritis (MeSH); Lymphoma, Mucosa-Associated Lymphoid Tissue (MeSH)
 METHODS & EQUIPMENT: Helicobacter pylori eradication therapy--complications, therapeutic method; reverse transcriptase-polymerase chain reaction--diagnostic method, polymerase chain reaction
 MISCELLANEOUS TERMS: B-cell clonality
 CONCEPT CODES:
 10052 Biochemistry methods - Nucleic acids, purines and pyrimidines
 10062 Biochemistry studies - Nucleic acids, purines and pyrimidines
 12504 Pathology - Diagnostic
 12512 Pathology - Therapy
 14004 Digestive system - Physiology and biochemistry
 14006 Digestive system - Pathology
 15002 Blood - Blood and lymph studies
 15006 Blood - Blood, lymphatic and reticuloendothelial pathologies
 24001 Neoplasms - Diagnostic methods
 24004 Neoplasms - Pathology, clinical aspects and systemic effects
 24008 Neoplasms - Therapeutic agents and therapy
 31000 Physiology and biochemistry of bacteria
 34502 Immunology - General and methods
 34508 Immunology - Immunopathology, tissue immunology
 36002 Medical and clinical microbiology - Bacteriology
 BIOSYSTEMATIC CODES:
 06210 Aerobic Helical or Vibrioid Gram-Negatives
 86215 Hominidae

3/9/17 (Item 5 from file: 34)
 DIALOG(R) File 34:SciSearch(R) Cited Ref Sci
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09259850 Genuine Article#: 385FU Number of References: 30
Title: Endoscopic characteristics of flow-grade gastric mucosa-associated lymphoid tissue lymphoma after eradication of Helicobacter pylori
 Author(s): Urakami Y (REPRINT) ; Sano T; Begum S; Endo H; Kawamata H; Oki Y
 Corporate Source: Urakami Gastro Clin,Dept Gastroenterol,2-2-2 Kitaokinosu/Tokushima 7700872//Japan/ (REPRINT); Urakami Gastro Clin,Dept Gastroenterol,Tokushima 7700872//Japan/; Univ Tokushima,Sch Med, Dept Pathol,Tokushima 770//Japan/; Univ Tokushima,Sch Dent, Dept Maxillofacial Surg,Tokushima 770//Japan/; Tokushima Prefectural Officers Clin,Tokushima//Japan/
 Journal: JOURNAL OF GASTROENTEROLOGY AND HEPATOLOGY, 2000, V15, N10 (OCT), P1113-1119
 ISSN: 0815-9319 Publication date: 20001000
 Publisher: BLACKWELL SCIENCE ASIA, 54 UNIVERSITY ST, P O BOX 378, CARLTON, VICTORIA 3053, AUSTRALIA
 Language: English Document Type: ARTICLE
 Geographic Location: Japan
 Journal Subject Category: GASTROENTEROLOGY & HEPATOLOGY
 Abstract: Background and Aims: It was recently reported that low-grade gastric lymphoma of mucosa-associated lymphoid tissue (MALT) was regressed by the eradication of Helicobacter pylori. The aim of this study was to confirm the effect of H. pylori eradication on low-grade gastric MALT lymphoma and to investigate the whitish mucosa that appeared with regression of the lesions.

Methods: Forty-seven H. pylori-positive patients with low-grade gastric MALT lymphoma were treated by using triple therapy. Biopsy specimens were histologically graded and B cell clonality was examined by using reverse transcription-polymerase chain reaction **before** and **after** eradication treatment. The relationship between the appearance of whitish mucosa and the degree of gastric gland loss was evaluated.

Results: Histologic regression was observed 2 months **after** eradication therapy in 42 of 47 patients. However, B cell monoclonality changed to polyclonality in only 23 patients **during** the follow-up period. The appearance of whitish mucosa in patients who showed histologic regression became more frequent as the degree of gastric gland loss increased ($P < 0.001$).

Conclusions: Most low-grade gastric MALT lymphoma histologically regressed **after** H. pylori eradication. The appearance of whitish mucosa **after** histologic regression reflected the degree of gastric gland loss. Whitish mucosa is an endoscopic characteristic and may be an endoscopic marker for regression of low-grade gastric MALT lymphoma.
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Descriptors--Author Keywords: B cell clonality ; empty lamina propria ; eradication ; gastric MALT lymphoma ; Helicobacter pylori ; mucosa-associated lymphoid tissue ; whitish mucosa

Identifiers--Keyword Plus(R): UNIDENTIFIED CURVED BACILLI; MALT-LYMPHOMA; CAMPYLOBACTER-PYLORI; **IMMUNE** -RESPONSE; REGRESSION; INFECTION; STOMACH

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- WYATT JI, 1988, V23, P44, SCAND J GASTROE S142

3/9/18 (Item 6 from file: 34)

DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

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09137979 Genuine Article#: 371AH Number of References: 34

Title: Comparison of invasive and noninvasive methods for the diagnosis and evaluation of eradication of Helicobacter pylori infection in children

Author(s): Yanez P; MadrazodelaGarza A; PerezPerez G; Cabrera L; Munoz O; Torres J (REPRINT)

Corporate Source: IMSS,CTR MED NACL SIGLO 21, HOSP PEDIAT, UNIDAD INVEST

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DEPT GASTROENTEROL/MEXICO CITY/DF/MEXICO/; VANDERBILT UNIV,SCH MED, DIV
INFECT DIS/NASHVILLE//TN/37212; HOSP PEDIAT MEXICO CITY,DEPT
PATOL/MEXICO CITY/DF/MEXICO/

Journal: ARCHIVES OF MEDICAL RESEARCH, 2000, V31, N4 (JUL-AUG), P415-421
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Abstract: Background, Acquisition of Helicobacter pylori infection occurs mainly **during** childhood. To study the events associated with H. pylori colonization in children it is important to have reliable diagnostic methods. Our objective was to validate invasive and noninvasive tests for diagnosis of H. pylori infection in children **before** and **after** antimicrobial treatment.

Methods. **Before** treatment, invasive rapid urease test (RUT) culture and histology, as well as the noninvasive carbon-13 urea breath test (C-13-UBT) and serology were validated in 59 children. The gold standard for H. pylori infection was any of three positives of the five tests. **After** antimicrobial treatment culture, histology, and C-13-UBT were validated in 43 children to determine eradication. The gold standard for eradication was negative in all three tests,

Results. For primary diagnosis, RUT was the most sensitive and specific test, followed by C-13-UBT, which performed better than serology, culture, and histology. Concordance tests also showed that RUT and C-13-UBT performed better. For determination of eradication, C-13-UBT and histology were better than culture, which showed poor sensitivity.

Conclusions. RUT performed better for primary diagnosis. However, as endoscopy might not be indicated in most children, C-13-UBT could be the test of choice for diagnosis of H. pylori infection both **before** and **after** eradication treatment, (C) 2000 IMSS. Published by Elsevier Science Inc.

Descriptors--Author Keywords: urease test ; histology ; serology ; gastrointestinal tract ; gold standard

Identifiers--KeyWord Plus(R): C-13-UREA BREATH TEST; RECURRENT ABDOMINAL-PAIN; **IMMUNE** -RESPONSE; CAMPYLOBACTER-PYLORIDIS; GASTRITIS; EPIDEMIOLOGY; TRANSMISSION; PREVALENCE; CHILDHOOD; COMMUNITY

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3/9/30 (Item 6 from file: 73)

DIALOG(R)File 73:EMBASE

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07392052 EMBASE No: 1998307445

Monitoring of anti-HP IgG during Helicobacter pylori eradication
 Antoljak N.; Vukadinovic M.V.; Zubcic A.; Topic E.

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 Zagreb Croatia

Clinical Laboratory (CLIN. LAB.) (Germany) 1998, 44/7-8 (529-532)

CODEN: CLLAF ISSN: 1433-6510

DOCUMENT TYPE: Journal; Article

LANGUAGE: ENGLISH SUMMARY LANGUAGE: ENGLISH; GERMAN

NUMBER OF REFERENCES: 13

There is no doubt that Helicobacter pylori infection results in inflammation of the gastric mucosa, called chronic superficial gastritis, which leads to peptic ulceration in some patients. Diagnostic tests are divided into two major categories: non-invasive tests and invasive methods using endoscopy. The aim of this study was to monitor anti-HP IgG by the ELISA Pyloriset EIA-G (Orion Diagnostica, Finland) during triple eradication therapy. Specific IgG were measured before treatment and then two, four and six months thereafter in plasma of 34 patients with positive history of dyspeptic illness. The mean percentage of titer decline was greatest two months after treatment (49%, $p < 0.001$). A statistically significant decrease continued after 4 and 6 months (66% and 78% of titer before therapy, respectively; $p < 0.005$). We found 94% of patients to be successfully seroconverted after 6 months monitoring. Contrary to other studies, our results showed that in 12% of the patients seroconversion occurred only after 6 months so that for such patients we recommend monitoring for a period of six months.

DEVICE BRAND NAME/MANUFACTURER NAME: Pyloriset EIA-G/orion/Finland

DEVICE MANUFACTURER NAMES: orion/Finland

DRUG DESCRIPTORS:

*immunoglobulin g antibody --endogenous compound--ec; *bacterium antibody
 --endogenous compound--ec

MEDICAL DESCRIPTORS:

*helicobacter pylori; *eradication therapy
 patient monitoring; chronic gastritis--etiology--et; chronic gastritis
 --therapy--th; peptic ulcer--complication--co; enzyme linked immunosorbent
 assay; endoscopy; seroconversion; human; male; female; clinical article;
 aged; adult; clinical trial; article

SECTION HEADINGS:

004 Microbiology: Bacteriology, Mycology, Parasitology and Virology
 005 General Pathology and Pathological Anatomy
 048 Gastroenterology

3/9/32 (Item 8 from file: 73)

DIALOG(R)File 73:EMBASE

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06830010 EMBASE No: 1997112511

Triple therapy for eradicating Helicobacter pylori and reducing the peptic ulcer recurrence rate. A 12-month follow-up study

ERADYKACJA **HELICOBACTER** PYLORI I ZMNIEJSZENIE WSKAZNIKA NAWROTU WRZODU TRAWIENNEGO 12 MIESIECY PO ZATOSOWANIU TERAPII POTROJNEJ

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(53-59)

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NUMBER OF REFERENCES: 31

Objectives: To determine the effect of H. pylori eradication on the natural history of peptic ulcer disease, and to evaluate the incidence of reinfection in patients where H. pylori was eradicated. Methods: Sixty eight H. pylori-positive patients (27 duodenal ulcer, 19 gastric ulcer, 16 gastritis, 6 **after** gastric resection) were treated with triple therapy (120 mg tripotassium dicitrato bismuthate q.d.s. for four weeks, 500 mg amoxycillin q.d.s. and 500 mg metronidazole t.d.s. for two weeks). H. pylori status (urease test, histology, culture) and serum **IgG antibodies** were determined **before** treatment, as well as 1 month and 12 months **after** finishing the therapy. The main measurement concerned the recurrence of peptic ulcer **after** one year in the eradicated and non-eradicated groups and the incidence of reinfection by H. pylori in the eradicated group **during** this follow-up period. No patient received maintenance therapy. Results: 1 month **after** the treatment the ulcers were healed in all of 46 patients with peptic ulcer disease and eradication of H. pylori infection was successful in 49 of 68 (72.1%) patients. **After** one year none of the 26 patients in the eradicated group experienced a peptic ulcer relapse. Two of the 20 patients (10%) in the non-eradicated group relapsed over the same period. Ten of the 49 patients (20.4%) in the eradicated group were reinfected **during** the follow-up period, so the eradication rate **after** one year was 57.4%. One year **after** the treatment **IgG** titres fell by 50% and more in 76.5% of the patients, irrespective of the success of bacterial eradication.

DRUG DESCRIPTORS:

*amoxicillin--drug dose--do; *amoxicillin--drug therapy--dt; *amoxicillin--pharmacology--pd; *bismuth citrate--drug dose--do; *bismuth citrate--drug therapy--dt; *bismuth citrate--pharmacology--pd; *metronidazole--drug dose--do; *metronidazole--drug therapy--dt; *metronidazole--pharmacology--pd

MEDICAL DESCRIPTORS:

*peptic ulcer--drug therapy--dt
adult; antibacterial activity; **antibody** titer; article; bacterial growth; drug effect; female; growth regulation; helicobacter pylori; human; major clinical study; male; recurrence risk; reinfection

CAS REGISTRY NO.: 26787-78-0, 61336-70-7 (amoxicillin); 57644-54-9, 813-93-4 (bismuth citrate); 39322-38-8, 443-48-1 (metronidazole)

SECTION HEADINGS:

037 Drug Literature Index
048 Gastroenterology

3/9/40 (Item 1 from file: 149)

DIALOG(R) File 149:TGG Health&Wellness DB(SM)

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Helicobacter pylori test-and- eradicate versus prompt endoscopy for management of dyspeptic patients: a randomised trial.(Statistical Data Included)

Lassen, Annmarie Touborg; Pedersen, Finn Moller; Bytzer, Peter; de Muckadell, Ove B Schaffalitzky

The Lancet, 356, 9228, 455

August 5,

2000

DOCUMENT TYPE: Statistical Data Included PUBLICATION FORMAT:

ABSTRACT: Treating dyspeptic patients in primary care with an *Helicobacter pylori* test-and-eradicate screening program is as safe and effective as use of endoscopy. Of 500 primary care patients who had dyspepsia involving two weeks or more of gastric pain, 250 were given test-and-eradicate treatment for *H. pylori* and the other 250 given endoscopic examination. A total of 28% of those tested for *H. pylori* were infected. **After** one year, there was no difference between the two groups in symptoms, quality of life, sick days, or doctor or hospital visits. There was less satisfaction with treatment in the *H. pylori* group.

TEXT:

Summary

Background Strategies based on screening for *Helicobacter pylori* to manage dyspeptic patients in primary care have been proposed, but the clinical consequences are unclear. We did a randomised trial to assess the efficacy and safety of a test-and-eradicate strategy compared with prompt endoscopy in the management of patients with dyspepsia.

Methods 500 patients presenting in primary care with dyspepsia (2 weeks of epigastric pain, no alarm symptoms) were assigned *H. pylori* testing plus eradication therapy or endoscopy. Symptoms, quality of life, patients' satisfaction, and use of resources were recorded **during** 1 year of follow-up.

Findings 250 patients were assigned test-and-eradicate, and 250 prompt endoscopy. The median age was 45 years and 28% were *H. pylori* infected. 1 year follow-up was completed by 447 patients. We found no differences in symptoms between the two groups (median registered days without dyspeptic symptoms=0.63 (IQR 0.27-0.81) in the test-and-eradicate group vs 0.67 (0.36- 0.86) in the prompt endoscopy group; mean difference 0.04 (95% CI 20.01-0.10), $p=0.12$). Nor did we find any difference in quality of life or numbers of sick-leave days, visits to general practitioners, or hospital admissions. In the test-and-eradicate group, 27 (12%) of the patients were dissatisfied with management, compared with eight (4%) in the endoscopy group ($p=0.013$). **After** 1 year, the use of endoscopies in the test-and-eradicate group was 0.40 times (95% CI 0.31-0.51) the use in the endoscopy group, the use of *H. pylori* tests increased by a factor of 8.1 (5.7- 13.1), the use of eradication treatments increased by a factor of 1.5 (0.9-2.7), and the use of proton-pump inhibitors was 0.89 (0.59-1.33) times the use in the endoscopy group. 43 (91% (80-98%)) of 47 peptic-ulcer patients would have been identified by endoscopy or treated by eradication therapy.

Interpretation A *H. pylori* test-and-eradicate strategy is as efficient and safe as prompt endoscopy for management of dyspeptic patients in primary care, although fewer patients are satisfied with their treatment.

Lancet 2000; 356: 455-60

Introduction

Dyspepsia is a common and costly problem, the main causes of which are functional dyspepsia ((greater than) 50%), peptic ulcer disease (20%), gastro-oesophageal reflux (20%), and gastric carcinoma ((less than) 2%). (1) However, the most cost-effective approach to the initial assessment and management of this disorder is controversial. Some clinicians advocate the use of early endoscopy in the assessment of all patients with dyspepsia, (2) whereas others argue that endoscopy should be reserved for patients with warning symptoms or those who have failed empirical medical management. (3,4)

Previous trials have shown that early endoscopy is more cost-effective than empirical management with histamine H2-receptor antagonists and leads to improved overall patient satisfaction, (5,6) but recognition that *Helicobacter pylori* is the major cause of peptic ulcers unrelated to non-steroidal anti-inflammatory drugs (NSAIDs) has begun to alter this approach. (7-10) In more developed countries, 25-50% of patients with dyspepsia are *H. pylori* positive, (11-14) of whom about a third to a half have peptic ulcer disease. (12,14,15)

Decision models have suggested that non-invasive testing for *H. pylori* with empirical eradication therapy for those who test positive may be more cost-effective than early endoscopy. (16-19) Eradication therapy is expected

to heal ulcers and resolve symptoms in most patients with peptic ulcer disease, and may lead to symptom resolution in a small number of infected patients with functional dyspepsia.(20-23) Results from two randomised trials in young dyspeptic patients with known H pylori status, and from one semirandomised study of young dyspeptic patients in primary care, support an H pylori test-and-eradicate strategy as cost-effective.(24-26) However, there are no published prospective, fully randomised trials that compare test-and-eradicate with prompt endoscopic assessment in a clinical setting for a complete group of dyspeptic patients.

Methods

Patients

65 general practitioners in Odense, Denmark (population 180000) agreed to refer dyspeptic patients to us for enrolment. Eligible patients had had dyspeptic symptoms (pain or discomfort in the epigastrium with or without heartburn, regurgitation, nausea, vomiting, or bloating) for at least 2 weeks of a nature or severity that made the physician suggest treatment or investigation of any kind. Exclusion criteria were age younger than 18 years; treatment with ulcer-healing drugs (except antacids) within the preceding month; any sign or suspicion of upper gastrointestinal bleeding, anaemia, or jaundice; unintended weight loss of more than 3 kg; any contraindication to endoscopy; previous surgery to the upper gastrointestinal tract; pregnancy; serious or terminal disorders; or suspected lack of cooperation. Patients were withdrawn from the study once enrolled if endoscopy revealed malignancy, or if pregnancy, a terminal illness, or unintended weight loss of more than 3 kg ensued.

All patients were interviewed, randomised, and investigated within 2 weeks of referral. All patients gave informed consent **before** inclusion, and the trial was approved by the local ethics committee.

To judge the rate of eligible patients not referred for the trial, the general practitioners registered all patients who consulted with dyspepsia in two 1-month periods.

Methods

Randomisation was done with tables of random numbers, and the results were kept in sealed, numbered envelopes. Patients were followed up for 1 year by means of interviews at entry, **after** 1 month and 1 year, and with diary cards for 1 week each month. All patients had a 13-carbon urea breath test **after** 1 year.

At entry, patients assigned H pylori testing were investigated by 13C-urea breath test (sensitivity 0.97, specificity 0.97, validated against histology and culture of gastric biopsy samples), and all patients were asked to discontinue any NSAID use. Infected patients received 30 mg lansoprazole twice daily, 500 mg metronidazole three times daily, and 1000 mg amoxicillin twice daily for 2 weeks. If symptoms had not improved at the 1 month visit (as judged by the patient on a four-grade Likert scale), or if symptoms recurred **during** follow up (judged by the patients to be as bad or worse compared with symptoms at entry), patients were offered endoscopy. H pylori-negative patients who had taken any amount of NSAID (including aspirin) **during** the previous month were examined by endoscopy. H pylori-negative patients not using NSAIDs who had reflux symptoms (predominantly heartburn or regurgitation) were treated with a proton-pump inhibitor (PPI; 30 mg lansoprazole daily) for 1 month. If successful, these patients continued PPI treatment on demand. If there was no improvement, patients were examined by endoscopy. H pylori-negative patients not using NSAIDs and without reflux symptoms were managed with reassurance and given advice of lifestyle modifications. In this group, 11 patients with a known symptomatic effect of acid inhibition were treated with PPI on demand.

Patients assigned prompt endoscopy were treated in accordance with the endoscopic findings, and all patients were asked to discontinue use of any NSAIDs. Patients with duodenal ulcers had eradication treatment followed by 2 weeks of PPI. Patients with gastric ulcers were treated according to H pylori status with either eradication treatment followed by 4 weeks of PPI, or with PPI for 6 weeks. Gastric ulcers were biopsied every 6 weeks until ulcers healed. Patients with reflux oesophagitis were given PPI for 8 weeks, followed by PPI on demand. Patients with normal findings or insignificant lesions (erythema or superficial erosions in the stomach or duodenum) at endoscopy were diagnosed as having functional dyspepsia, and were managed with reassurance and given advice on lifestyle modifications. In this group, 12 patients with a known symptomatic effect of acid inhibition were treated with PPI on demand.

Each month, a diary was mailed to the patient just **before** a 1-week registration period. Symptoms were graded daily (no, mild, moderate, or severe influence on lifestyle of any dyspeptic symptom). Use of medication, sick-leave days, and visits to a general practitioner in the previous month were also registered. At entry, and **after** 1 month and 1 year, patients rated overall influence of dyspeptic symptoms on a visual analogue scale and completed a validated symptom questionnaire--the gastrointestinal symptoms rating scale.(27) Quality of life was estimated by a self-administered, validated questionnaire--the psychological general well-being index.(28) **After** 1 month and 1 year, patients were asked to compare their symptoms with those at entry (none/improvement/no change/worse), and to state their satisfaction with medical care on a four-grade scale (very satisfied/satisfied/dissatisfied/very dissatisfied).

After 1 year, the total number of endoscopies, H pylori tests, eradication treatments, and PPI consumption were registered. Sick-leave days, and visits to general practitioners were counted from the diaries. Information on visits to outpatient clinics and hospital admissions was obtained from hospital databases. The main outcome measure was rate of well-being days (proportion of days without dyspeptic symptoms) registered in the diaries **during** 1 year follow-up. With a type I error of 5% and a power of 90%, the study could detect a true 0.10 difference in the mean rate in each group (two-sample t test). Secondary outcome measures were use of resources such as endoscopies (90% power to detect a 0.25 difference in mean use), H pylori tests (0.15 difference in mean use), eradication treatments (0.15 difference in mean use), and use of PPIs (28DDD difference in mean use).

Statistical analysis

All statistical analyses were two sided. Group comparisons of rates and proportions were assessed by the χ^2 test for trends or the Mann-Whitney test, as appropriate.

Results

576 patients were referred, 76 of whom were not enrolled (symptoms resolved (five), lack of consent (13), pregnancy (two), need of an interpreter (22), use of antisecretory medication in the past month (14), major weight loss or concomitant disease (18), or other reasons (two)). Registrations from the two 1-month periods in which all dyspeptic patients in primary care were registered showed that 88 (85%) of 104 eligible patients were referred and included in the study.

500 patients were randomised: 250 to each management group. 15 patients (six in the test-and-eradicate group) were withdrawn **during** follow-up. One patient in this group died; the cause of death was unrelated to dyspepsia. 245 (98%) of the patients in the test-and-eradicate group and 239 (96%) of the patients in the endoscopy group returned for the 1-month interview. 223 (89%) of the patients in the test-and-eradicate group and 224 (90%) of the patients in the endoscopy group returned for the 1-year interview (figure 1). The two groups were well matched, except that patients assigned prompt endoscopy had a longer history of dyspepsia (table 1).

Of the 250 patients in the test-and-eradicate group, 64 (26%) were H pylori positive, 40 (16%) were H pylori negative but had used NSAID in the last month, and 50 (20%) had reflux symptoms, negative H pylori status, and no NSAID use. 96 (38%) were H pylori negative with no NSAID use and no reflux symptoms. 100 (40%) of the patients in the test-and-eradication group had an endoscopy. 38 (59%) of the H pylori-positive patients had endoscopy **during** follow-up due to no symptomatic effect of eradication therapy, or symptom relapse; three patients were found to have peptic ulcer, 15 had reflux oesophagitis, and 20 had a normal endoscopy. All 40 H pylori-negative patients who had used NSAIDs had endoscopy at entry; five were found to have peptic ulcer, 13 reflux oesophagitis, and 22 a normal endoscopy. 14 of the 50 H pylori-negative patients with reflux symptoms had endoscopy due to no effect of PPI treatment two were found to have reflux oesophagitis, 12 had normal endoscopy. Eight of the 96 H pylori-negative patients without use of NSAIDs and without reflux symptoms were referred for endoscopy by their general practitioners; they all had a normal endoscopy. No patients in the test-and-eradicate group had gastric or oesophageal cancer diagnosed **during** follow up.

Of the 250 patients assigned prompt endoscopy, two patients did not attend, 22 (9%) had a duodenal ulcer, 25 (10%) a gastric ulcer, 70 (28%) reflux oesophagitis, 129 (52%) had a normal endoscopy or insignificant

lesions, and two (1%) had gastric cancer (a 76-year-old man (H pylori positive) with adenocarcinoma, and a 22-year-old man (H pylori negative) with malignant lymphoma). When patients in the endoscopy group were categorised by H pylori status, use of NSAID, and reflux symptoms, we found that 76 (31%) were H pylori positive, 28 (11%) were H pylori negative but had used NSAIDs in the previous month, 46 (19%) had reflux symptoms, negative H pylori status and no NSAID use, and 97 (39%) were H pylori negative with no NSAID use and no reflux symptoms. 43 (91%) of 47 patients with peptic ulcer were H pylori positive or had used NSAIDs (figure 2).

We found no difference between the groups in the proportion of days with and without dyspeptic symptoms registered in the diaries. The median proportion of days without dyspeptic symptoms was 0.63 (IQR 0.27-0.81) in the test-and-eradicate group versus 0.67 (0.36-0.86) in the prompt endoscopy group ($p=0.12$; figure 3). There were no differences between the two groups in scores on the gastrointestinal symptoms rating scale and the visual analogue scale, or quality of life at entry, **after** 1 month, or at 1 year (table 2). At 1 month, significantly more patients in the prompt endoscopy group reported improvement of symptoms, but **after** 1 year there were no differences between the groups. Fewer patients in the test-and-eradicate group were satisfied with management, both **after** 1 month and **after** 1 year (table 2).

We found no differences in the mean number of visits to general practitioners or outpatient clinics, hospital admissions, and sick-leave days. **After** 1 year, the use of endoscopies in the test-and-eradicate group was 0.40 times (95% CI 0.31-0.51) that in the prompt endoscopy group, equalling 0.75 (0.62-0.88) fewer endoscopies per patient in the test-and-eradicate group than the prompt endoscopy group. Use of PPI was lower in the test-and-eradicate group than the endoscopy group, whereas the use of H pylori tests and eradication treatments was higher in the former than the latter (table 3).

Severe side-effects leading to discontinuation of treatment were experienced by 14 (13%) of 104 patients who were given eradication therapy. Eradication was achieved in 99 (87%) of all patients starting eradication therapy.

At entry, all patients had epigastric pain or discomfort, but 159 (84 in the test-and-eradicate group) had heartburn or regurgitation as the dominant symptom. The findings for the whole study group applied equally to the subset of patients with or without reflux symptoms, except that among patients with reflux symptoms we found no difference in satisfaction with management between the two groups. 255 of the study participants (133 in the test-and-eradicate group) were younger than 45 years. The findings for the whole study group applied equally to these patients, except for satisfaction with management, where we found no difference between the two groups.

Discussion

We found that the test-and-eradicate strategy was as efficient as prompt endoscopy with regard to symptoms, quality of life, sick-leave days, visits to general practitioners, and hospital admissions. Notably, the strategy was found to be as safe as prompt endoscopy, since 91% of all peptic-ulcer patients were either treated with eradication therapy or subsequently identified by endoscopy.

84% of all eligible patients in primary care were included in the trial. We therefore regard the results to be applicable to primary-care patients despite the fact that randomisation and follow-up took place in a university clinic. However, the results regarding patients' satisfaction might have been modified by the referral, owing to a possible introduced expectation bias either towards or against endoscopy among some of the patients. The main entry criterion was epigastric pain or discomfort, which was reported by all patients. However, 32% of the patients in the trial had heartburn as their dominant symptom. Even so, we found no differences in the outcomes of the trial either for these patients, or for patients without dominant reflux symptoms, compared with the whole study group. Since the power of the trial was only slightly reduced in these subanalyses (75% for patients with reflux, and 85% for patients without reflux vs 90% in the whole study group), we believe that the overall conclusions are also valid for these patients. Two other trials of young dyspeptic patients without reflux symptoms support a test-and-eradicate strategy as cost-effective. (24,26) The outcome of a trial which randomised only young H pylori-negative patients also supports the strategy. (25) Thus, three other

trials are in accordance with results from our study, in which dyspeptic patients with or without concomitant reflux symptoms and in an age range from 18 to 88 years were included.

12% of the patients assigned test-and-eradicate were not satisfied. In daily practice, these patients present a special challenge to their general practitioner. They may continue to demand invasive and costly tests such as endoscopy, making the H pylori test-and-eradicate strategy less efficient. But since the number of dissatisfied patients is small, the total effect is expected to be marginal, at least in a population similar to that investigated.

One of the potential drawbacks of introducing an H pylori test-and-eradicate strategy is the use of eradication therapy in dyspeptic patients without peptic ulcer disease, since patients with reflux oesophagitis and functional dyspepsia will have no or very little symptomatic benefit from H pylori eradication. Some may even argue that H pylori eradication may harm these patients by making them more resistant to PPI treatment. On the other hand, in some of these patients, development of peptic ulcer disease or gastric cancer may have been averted.

A main concern regarding an H pylori test-and-eradicate strategy is the possibility of missing gastric cancer at an early stage. Therefore recommendations for H pylori-based management strategies are restricted to young patients without possible symptoms of gastric cancer. (7-10) The power of our trial is insufficient to judge the appropriateness of an age limit, since we found only two cancers in the control group. Another important safety aspect relates to the proper management of ulcer patients relying only on the risk factors of H pylori infection and NSAID use. 47 ulcer patients were identified in the endoscopy group; four of these patients (9%) were H pylori-negative and had not used NSAIDs, indicating that 91% of all ulcer patients managed by an H pylori test-and-eradicate strategy will be either cured or identified by endoscopy.

The trial adds important information on dyspeptic symptoms, quality of life, patients' satisfaction, and use of resources to existing theoretical analysis concerning economic aspects of the management of dyspeptic patients. This trial was not, however, designed to estimate the cost-effectiveness of the management strategies. Such an analysis may not be useful owing to large variations in costs and differences in health-care systems. The cost-effectiveness of management options in dyspepsia are affected substantially by the cost of gastroscopy. Published costs for gastroscopy differ widely, ranging from US\$53 in Germany to US\$1180 in the USA. Even within the same country, cost estimates published in the same year differ by 400%. (29) The use of resources summarised in table 3 will allow the reader to make their own assessments about the cost-effectiveness of the two strategies in their own clinical settings. In a public-health perspective, availability of resources will influence the choice of strategy. If endoscopy is provided on a short waiting list, the introduction of an H pylori test-and-eradicate strategy may not be relevant. However, if access to endoscopy is restricted, an H pylori test-and-eradicate strategy might be a safe and efficient alternative.

In view of the rapidly changing epidemiology of H pylori infection, the long-term effects of this strategy should be monitored closely, especially if the prevalence of H pylori in ulcer disease continues to decline. (30)

Contributors

A T Lassen was responsible for recruiting patients, doing tests and endoscopies, recording clinical observations, and analysing data. F M Pedersen was involved in recruitment of patients and data collection. O B Schaffalitzky de Muckadell and P Bytzer were involved in the original design and instigation of the study. All investigators contributed to the writing of the paper.

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	Test-and-eradicate group (n=250)	Prompt endoscopy group (n=250)
Demographics		
Median (range) age (years)	44 (18-88)	47 (19-84)
Men/women	105/145	125/125
History		
Median (range) history of dyspepsia (years)	3.2 (0-50)	7.9 (0-70)
Median (range) length of present episode (months)	2.8 (0-180)	2.5 (0-120)
Median (range) sick-leave days in preceding month	0 (0-30)	0 (0-30)
NSAID use in past month	53 (21%)	48 (19%)
H pylori positive	64 (26%)	77 (31%)
Smoker	114 (46%)	117 (47%)
Previous PPI or H2-blocker therapy	107 (43%)	99 (40%)
Previous H pylori test	0	3 (1%)
Previous endoscopy	70 (28%)	68 (27%)
Previous peptic ulcer	20 (8%)	22 (9%)
Previous oesophagitis	11 (21%)	13 (5%)
Effect of dyspepsia on lifestyle		
No/minimal	15 (6%)	15 (6%)
Minor influence	126 (54%)	121 (48%)
Moderate influence	99 (40%)	110 (44%)
Debilitating symptoms	0	4 (2%)
Dominant symptom complex		
Heartburn and/or regurgitation	84 (34%)	75 (30%)
Epigastric pain	86 (34%)	101 (40%)
Nausea	24 (10%)	25 (10%)
Bloating	56 (22%)	49 (20%)

NSAID=non-steroidal anti-inflammatory drug. PPI=proton-pump inhibitor.

Table 1: Baseline characteristics
Entry

	Test-and- eradicate group (n=250)	Prompt endoscopy group (n=250)	p
Median (IQR) score on rating scale			
Gastrointestinal symptoms rating scale(*)	2.4 (1.9-3.0)	2.3 (1.9-2.8)	0.22
Visual analogue scale()	50 (34-66)	50 (35-62)	0.79
Psychological general well-being index(c)	94 (79-106)	96 (81-107)	0.31
Improvement(sections)			
No symptoms
Improved
Unchanged
Worse
Satisfaction with management(para)			
Very satisfied
Satisfied
Dissatisfied
	1 month Test-and- eradicate group (n=245)	Prompt endoscopy group (n=239)	p
Median (IQR) score on rating scale			
Gastrointestinal symptoms rating scale(*)	1.7 (1.4-2.2)	1.7 (1.3-2.3)	0.97
Visual analogue scale()	20 (6-45)	17 (5-41)	0.36
Psychological general well-being index(c)	108 (95-116)	107 (91-118)	0.52
Improvement(sections)			
No symptoms	48 (20%)	68 (28%)	0.005
Improved	120 (49%)	117 (49%)	
Unchanged	62 (25%)	46 (19%)	
Worse	15 (6%)	8 (3%)	
Satisfaction with management			
Very satisfied	132 (54%)	167 (70%)	(less than)
			0.001
Satisfied	77 (31%)	66 (28%)	
Dissatisfied	36 (15%)	6 (3%)	
	1 year Test-and- eradicate group (n=223)	Prompt endoscopy group (n=224)	p
Median (IQR) score on rating scale			
Gastrointestinal symptoms rating scale(*)	1.7 (1.3-2.2)	1.7 (1.3-2.1)	0.51
Visual analogue scale()	15 (4-35)	14 (4-33)	0.36
Psychological general well-being index(c)	108 (98-117)	110 (99-117)	0.38
Improvement(sections)			
No symptoms	50 (22%)	55 (25%)	0.66
Improved	128 (57%)	114 (51%)	
Unchanged	38 (17%)	46 (21%)	

Worse	7 (3%)	8 (4%)	
Satisfaction with management			
Very satisfied	124 (56%)	139 (62%)	0.013
Satisfied	72 (32%)	77 (34%)	
Dissatisfied	27 (12%)	8 (4%)	

(*) Decreasing value means decreasing symptoms. () 0=no symptoms, 100=worst possible symptoms. (c) Increasing value means increasing quality of life.

(sections) Patients' statement of overall improvement of symptoms compared with symptoms at entry.

Table 2: Symptoms, quality of life, and patients' satisfaction with management at entry, **after** 1 month, and **after** 1 year
Test-and-eradicate group

Mean (95% CI) diagnostic tests	
Endoscopies	0.5 (0.41-0.59)
H pylori tests	1.13 (1.09-1.18)
Mean (95% CI) eradication therapies	0.26 (0.20-0.32)
Mean (95% CI) treatments (DDD)	
PPI use	52.2 (40.9-63.4)
H2 blockers(*)	0.98 (0.49-1.47)
Antacids(*)	3.42 (2.40-4.07)
Mean (95% CI) visits to outpatient clinics	
Dyspepsia-related	0.08 (0.02-0.14)
Other	1.10 (0.78-1.43)
Mean (95% CI) days in hospital	
Dyspepsia-related	0.19 (0.04-0.35)
Other	1.73 (0.99-2.48)
Mean (95% CI) sick-leave days	
Dyspepsia-related	2.05 (1.42-2.68)
Other	12.14 (6.98-17.3)
Mean (95% CI) visits to GPs	
Dyspepsia-related	0.98 (0.71-1.25)
Other	3.83 (3.15-4.51)
Prompt endoscopy group	
Mean (95% CI) diagnostic tests	
Endoscopies	1.25 (1.16-1.34)
H pylori tests	0.14 (0.09-0.19)
Mean (95% CI) eradication therapies	0.17 (0.12-0.22)
Mean (95% CI) treatments (DDD)	
PPI use	58.7 (47.8-69.6)
H2 blockers(*)	0.70 (0.35-1.15)
Antacids(*)	2.25 (1.67-2.85)
Mean (95% CI) visits to outpatient clinics	
Dyspepsia-related	0.09 (0.03-0.15)
Other	1.38 (0.94-1.82)
Mean (95% CI) days in hospital	
Dyspepsia-related	0.32 (0.04-0.60)
Other	1.83 (0.81-2.86)
Mean (95% CI) sick-leave days	
Dyspepsia-related	2.89 (1.95-3.83)

Other	10.84 (7.04-14.65)
Mean (95% CI) visits to GPs	
Dyspepsia-related	0.66 (0.48-0.85)
Other	3.06 (2.57-3.15)
	Difference
Mean (95% CI) diagnostic tests	
Endoscopies	20.75 (20.88 to 20.62)
H pylori tests	0.99 (0.93-1.06)
Mean (95% CI) eradication therapies	0.09 (0.02-0.17)
Mean (95% CI) treatments (DDD)	
PPI use	26.53 (222.15-9.10)
H2 blockers(*)	0.28 (20.38-0.94)
Antacids(*)	0.98 (20.05-2.00)
Mean (95% CI) visits to outpatient clinics	
Dyspepsia-related	20.01 (20.09-0.08)
Other	20.28 (20.82-0.27)
Mean (95% CI) days in hospital	
Dyspepsia-related	20.13 (20.45-0.19)
Other	20.10 (21.36-1.16)
Mean (95% CI) sick-leave days	
Dyspepsia-related	20.84 (21.97-0.29)
Other	1.30 (25.10-7.70)
Mean (95% CI) visits to GPs	
Dyspepsia-related	0.32 (20.02-0.65)
Other	0.77 (20.06-1.61)
	Ratio
Mean (95% CI) diagnostic tests	
Endoscopies	0.40 (0.31-0.51)
H pylori tests	8.07 (5.74-13.11)
Mean (95% CI) eradication therapies	1.53 (0.91-2.67)
Mean (95% CI) treatments (DDD)	
PPI use	0.89 (0.59-1.33)
H2 blockers(*)	1.40 (0.42-5.88)
Antacids(*)	1.44 (0.84-2.43)
Mean (95% CI) visits to outpatient clinics	
Dyspepsia-related	0.89 (0.13-4.67)
Other	0.80 (0.43-1.52)
Mean (95% CI) days in hospital	
Dyspepsia-related	0.59 (0.07-8.75)
Other	0.95 (0.35-3.06)
Mean (95% CI) sick-leave days	
Dyspepsia-related	0.71 (0.37-1.37)
Other	1.12 (0.45-2.46)
Mean (95% CI) visits to GPs	
Dyspepsia-related	1.48 (0.84-3.15)
Other	1.25 (1.00-1.75)
	p
Mean (95% CI) diagnostic tests	
Endoscopies	(less than) 0.0001
H pylori tests	(less than) 0.0001

Mean (95% CI) eradication therapies	0.009
Mean (95% CI) treatments (DDD)	
PPI use	0.03
H2 blockers(*)	0.54
Antacids(*)	0.27
Mean (95% CI) visits to outpatient clinics	
Dyspepsia-related	0.65
Other	0.83
Mean (95% CI) days in hospital	
Dyspepsia-related	0.63
Other	0.65
Mean (95% CI) sick-leave days	
Dyspepsia-related	0.87
Other	0.26
Mean (95% CI) visits to GPs	
Dyspepsia-related	0.41
Other	0.06

(*) Registered in 12/52 weeks of the observation period.

Table 3: Mean (per patient) use of resources
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Eradication of Helicobacter pylori in functional dyspepsia: randomised
double blind placebo controlled trial with 12 months' follow up.
Talley, Nicholas J; Janssens, Jef; Lauritsen, Karsten; Racz, Istvan;
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ABSTRACT: The bacteria Helicobacter pylori does not seem to be involved with the cause of symptoms in functional dyspepsia, and the elimination of H. pylori does not seem to relieve those symptoms. Dyspepsia is a combination of gastrointestinal discomforts including heartburn and bloating, but most patients do not have any peptic ulceration or other disease associated with H. pylori. Of 278 patients treated, H. pylori was eliminated in 113. At the followup after 12 months, symptoms remained largely unrelieved, there was not enough appreciable difference between those treated and those untreated to attribute any cause to H. pylori.
TEXT:

Abstract

Objectives To determine whether eradication of Helicobacter pylori relieves the symptoms of functional dyspepsia.

Design Multicentre randomised double blind placebo controlled trial.
Subjects 278 patients infected with who had functional dyspepsia.

Setting Predominantly secondary care centres in Australia, New Zealand, and Europe.

Intervention Patients randomised to receive omeprazole 20 mg twice daily, amoxicillin 1000 mg twice daily, and clarithromycin 500 mg twice

daily or placebo for 7 days. Patients were followed up for 12 months.

Main outcome measures Symptom status (assessed by diary cards) and presence of (assessed by gastric biopsies and (13)C-urea breath testing using urea labelled with carbon-13).

Results H pylori was eradicated in 113 patients (85%) in the treatment group and 6 patients (4%) in the placebo group. At 12 months follow up there was no significant difference between the proportion of patients treated successfully by intention to treat in the eradication arm (24%, 95% confidence interval 17% to 32%) and the proportion of patients treated successfully by intention to treat in the placebo group (22%, 15% to 30%). Changes in symptom scores and quality of life did not significantly differ between the treatment and placebo groups. When the groups were combined, there was a significant association between treatment success and chronic gastritis score at 12 months; 41/127 (32%) patients with no or mild gastritis were successfully treated compared with 21/123 (17%) patients with persistent gastritis (P = 0.008).

Conclusion No convincing evidence was found that eradication of relieves the symptoms of functional dyspepsia 12 months **after** treatment.

Introduction

Most patients with dyspepsia do not have any peptic ulceration or other disease(1-4); they are classed as having functional dyspepsia. About 50% of patients with functional dyspepsia have co-existent Helicobacter pylori gastritis, (3 5-7) but it is unclear whether H pylori causes symptoms in the absence of peptic ulceration. (8-10)

Carefully conducted trials should be able to determine whether or not H pylori is a cause of functional dyspepsia, as symptoms would be expected to abate when H pylori was eradicated. (11) Previous trials, however, have been conflicting and the methods have been generally suboptimal. (8 9) Moreover, few studies have tested whether eradication of H pylori improves dyspepsia long term. As it may take at least 12 months for gastritis, as confirmed by histology, to return to normal, prolonged follow up may be required to observe resolution of symptoms in functional dyspepsia. (12 13)

We postulated that H pylori is a direct cause of around 20% of cases of functional dyspepsia. To test this hypothesis, we conducted a controlled trial. The study protocol was approved by the appropriate ethics committees, and written informed consent was obtained from the participants.

Subjects and methods

Overall, 278 consecutive patients were recruited from 40 centres in Australia, New Zealand, and nine European countries; 244 patients (89%) were from secondary care. The remaining 31 patients (11%) were from primary care and were recruited only from the United Kingdom. Twenty centres recruited six or more patients.

Protocol

Study population--Dyspepsia was defined as pain or discomfort centred in the upper abdomen. (1) We enrolled adult patients with dyspepsia for at least 3 months, normal endoscopic findings, and a positive result for H pylori on a screening test (Helisal, Cortecs Diagnostics, UK). Patients with oesophagitis (any mucosal break), Barrett's oesophagus, gastric or duodenal ulceration, duodenal erosions, malignancy, more than five gastric erosions, or alarm symptoms were excluded. (H.sub.2) receptor antagonists, prostaglandins, or prokinetics **during** the 7 days **before** enrolment, or proton-pump inhibitors, antibiotics, or bismuth **during** the 30 days **before** enrolment, were not permitted. Patients with documented peptic ulcer disease or gastro-oesophageal reflux disease were excluded.

Run-in period-- **After** endoscopy, patients were required to fill out a diary card with scores for their symptoms **during** a 7 day run-in period. Only patients who had at least 3 days of at least moderate dyspepsia symptoms were randomised. No study drug was dispensed **during** the run-in.

Treatment period--Patients underwent a breath test using urea labelled with carbon-13 at the randomisation visit. They were randomised to receive either omeprazole 20 mg twice daily, amoxicillin 1000 mg twice daily, and clarithromycin 500 mg twice daily or placebo for 1 week. If patients had taken at least 12 out of 14 doses of drug or placebo they were considered to be compliant; no patients were withdrawn from the study because of poor compliance.

Follow up period--The patients were followed up 1, 3, 6, 9, and 12 months **after** cessation of treatment. Diary cards (filled out the week **before** each visit) were collected at each visit, quality of life forms

were filled out by the patients at the 6 and 12 month visit, and a urea breath test and upper endoscopy were performed at the 3 and 12 month visits. A weak antacid (with a neutralising capacity of around 13 mmol of hydrochloric acid per tablet) was dispensed at each visit and its consumption was recorded. **During** follow up, patients could receive treatment for dyspeptic symptoms from their doctor but all drugs used were recorded.

Primary outcome measures

Patients recorded the severity of their dyspepsia symptoms on diary cards using a validated Likert scale comprising 7 grades: none, minimal, mild, moderate, moderately severe, severe, very severe. (14)

At each endoscopic evaluation, two antral and two corpus biopsy specimens were obtained. Specimens were stained with haematoxylin and eosin and with the Steiner silver method.

The biopsies were histologically graded. (15) All specimens were reviewed by an experienced gastrointestinal pathologist blinded to the treatment group. Urea breath testing was performed using a standard validated European protocol. (16)

At pre-entry, two test results for *H pylori* had to be positive; by screening test (Helisal rapid blood test or a rapid urease test) and by either urea breath testing or histological assessment.

After treatment, *H pylori* status was assessed at 3 and 12 months. If any of the gold standard assessments (urea breath test or histology) were positive, patients were considered to be positive for *H pylori*. If only one test result was available, the outcome of that test determined the *H pylori* status.

Secondary outcome measures

The gastrointestinal symptom rating scale was used to score dyspepsia symptoms. This validated instrument measures symptoms including abdominal pain. (17 18) The psychological general well being index was used to score the patients' quality of life. This validated instrument measures subjective well being. (18-20)

Patients were subdivided into symptom subgroups on the basis of their responses to the gastrointestinal symptom rating scale. Ulcer-like dyspepsia was defined as at least moderate stomach pain and hunger pain in the week **before** follow up. Dysmotility-like dyspepsia was defined by two or more of at least moderate bloating, nausea, stomach rumblings, or belching in the week **before** follow up. The subgroups were not mutually exclusive.

Statistical analyses

Patients were excluded from the intention to treat analysis who were negative for at pre-entry or who were without any assessment of treatment efficacy **after** randomisation (fig 1). The treatment groups were compared for symptom relief with a Mantel-Haenszel test stratified by country and for healing of gastritis with a Mantel-Haenszel test stratified by baseline gastritis.

(Figure 1 ILLUSTRATION OMITTED)

Patients who reported on the diary card no more than minimal dyspepsia symptoms **during** any of the 7 days **before** the 12 month visit were considered a priori to be a treatment success.

Chronic gastritis was considered healed when both antrum and corpus specimens had an inflammation score of zero. (15)

The treatment groups were compared for change in the total score of the gastrointestinal symptom rating scale and psychological general well being index from the first visit to the last visit in the study, using the baseline value as a covariate in an analysis of covariance model.

With 275 patients, the power of the study was 94% provided the true proportions of responders was 20% and 40% in the two groups (assuming an a level of 0.05 based on a two sided (chi square) test). The placebo response was based on data for symptom turnover. (21)

Randomisation was in blocks of four in proportions of 1:1 according to a computer generated list.

Identical placebos were used. Investigators and patients were blinded to all data, including *H pylori* assessments **after** randomisation, until the study was fully completed.

Results

One hundred and thirty five patients (52 men) were randomised to treatment and 143 patients (48 men) were randomised to placebo (fig 1). Three patients (two in the treatment group and one in the placebo group)

were withdrawn from the analysis because of unavailability of data **after** randomisation.

The two groups were well balanced for demographic and clinical features (table 1).

Table 1 Baseline data of patients allocated omeprazole, amoxicillin, and clarithromycin or placebo. Values are number (percentage) of patients, unless stated otherwise

Baseline characteristic	Treatment group (n=133)	Placebo group (n=142)
Mean age (years) (SD)	51 (14)	49 (13)
Male	51 (38)	47 (33)
Ethnic origin:		
White	130 (98)	140 (99)
Smoker	26 (20)	39 (27.5)
Alcohol use	50 (38)	51 (36)
Duration of dyspepsia >1 year	104 (78)	106 (75)

Analysis

Eradication of H pylori and healing of gastritis Both urea breath testing and histology results were available for 237 patients (86%). At 12 months, 113 patients (85%) in the treatment arm had been successfully cured of H pylori infection compared with 6 patients (4%) in the placebo group. However, 108 patients (81%) in the treatment group had no or mild chronic gastritis at 12 months compared with 18 patients (13%) in the placebo group (table 2). Overall, 98% of patients consumed at least 12 of 14 doses in both groups.

Table 2 Main study outcomes 12 months **after** treatment with omeprazole, amoxicillin, and clarithromycin or placebo. Values are number (percentage) unless stated otherwise

Variable	Type of analysis	Treatment group
Treatment success(*)	Intention to treat	32 (24) n=133
Treatment success(*)	Per protocol	27 (28) n=95
Active chronic gastritis grade 0((dagger))	intention to treat	94 (71)
Chronic gastritis grade 0((double dagger))	Intention to treat	25 (19)
Chronic gastritis grades 0 and 1((double dagger))	Intention to treat	108 (81) n=133

Variable	Placebo group	% difference (95% CI)
Treatment success(*)	31 (22) n=142	2 (-8 to 12)
Treatment success(*)	29 (29) n=100	-1 (-13 to 12)
Active chronic gastritis grade 0((dagger))	4 (3)	69 (61 to 77)
Chronic gastritis grade 0((double dagger))	1 (1)	18 (12 to 25)
Chronic gastritis grades 0 and 1((double dagger))	18 (13) n=142	68 (59 to 76)

Variable	P value
Treatment success(*)	0.7
Treatment success(*)	0.9
Active chronic gastritis grade 0((dagger))	<0.001
Chronic gastritis grade 0((double dagger))	<0.001
Chronic gastritis grades 0 and 1((double dagger))	<0.001

(*) No or only minimal pain or discomfort centred in upper abdomen over 7 days **before** 12 month visit.

((dagger)) Presence of polymorphonuclear cells.

((double dagger)) Presence of mononuclear cells.

Symptom relief

By an intention to treat analysis, 32 patients (24%) in the treatment group and 31 patients (22%) in the placebo group were successfully treated (minimal or no dyspepsia) at 12 months (table 2). There was no significant difference in treatment success among those who were negative for *H pylori* (35, 29%) and those who remained positive for *H pylori* (28, 21%). At the 12 month follow up, no dyspepsia symptoms were reported by 20 patients (15%) in the treatment group and 16 patients (11%) in the placebo group. A similar proportion of patients in each treatment arm had no or minimal dyspepsia symptoms at each follow up (fig 2). The mean symptom score was not significantly different at each time point (fig 3). There was no inhomogeneity among countries (Breslow-Day test, P is greater than) 0.20).

(Figures 2-3 ILLUSTRATION OMITTED)

Mean antacid consumption over 12 months did not differ significantly between treatment (0.53 tablets per day) and placebo groups (0.65). Five patients (one in the treatment group and four in the placebo group) had treatment success according to the diary cards but were considered treatment failures in the analysis because they took a gastrointestinal drug other than antacid within 2 weeks of the 12 month visit.

In the ulcer-like dyspepsia group, treatment success was reported by 17/68 patients (25%) in the treatment group and 12/58 patients (21%) in the placebo group. The corresponding results for dysmotility-like dyspepsia were 14/78 patients (18%) in the treatment group and 13/73 patients (18%) in the placebo group.

The change from baseline to last visit between the treatment groups was not significant for either the psychological general well being index or the gastrointestinal symptom rating scale (fig 4).

(Figure 4 ILLUSTRATION OMITTED)

Gastritis scores and symptom relief--There was no association between the severity of symptoms at baseline and gastritis scores on initial biopsies. Patients at follow up were subdivided regardless of treatment into those with a chronic gastritis score of 0 or 1 (none or mild gastritis) and those with a score of 2 or 3 (moderate or severe gastritis) in a secondary analysis. At the 12 month follow up, 41/127 patients (32%) with no or mild gastritis were treatment successes (no or minimal dyspepsia) compared with 21/123 patients (17%) with moderate or severe gastritis ($P=0.008$). This association was not explained by age. Of the 41 patients with none or mild gastritis at follow up, only nine had received placebo (of whom only one had complete resolution of gastritis and eight had mild gastritis).

Discussion

Few large trials have rigorously evaluated the role of *H pylori* eradication in functional dyspepsia, and the results are conflicting.(22 23) We found no convincing evidence that successful eradication of *H pylori* infection relieves or reduces symptoms in patients with functional dyspepsia over 12 months.

Trial design issues

We aimed to overcome previous methodological limitations.(8 9) In particular, the outcome measures were valid and responsive to change.(14 17-20) Scrupulous attention was paid to blinding of patients and investigators. Prospective assessment of symptoms reduced the issue of recall bias.(9)

Predictors of symptom relief

A persistent inflammatory response could promote the development of dyspepsia.(11) We observed an association between healing of chronic gastritis and symptom relief but this secondary analysis requires confirmation.

A few studies have observed that treatment response was limited to those patients with ulcer-like dyspepsia, but no link between dyspepsia subgroups and *H pylori* eradication was evident in the present study.(13 24) Although our results may be generalisable to secondary care, we cannot exclude the possibility that such patients have more resistant symptoms than those in primary care where trials are needed.

Management implications--A popular management strategy in otherwise healthy young patients with uninvestigated dyspepsia is to non-invasively test for *H pylori* and to treat all infected cases.(25) Although controversial, such a strategy may have a number of potential benefits.(26) On the basis of our results, however, only a minority who are treated would be likely to gain long term symptomatic relief, because most infected

patients with dyspepsia have functional dyspepsia rather than peptic ulcer disease. (25)

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Randomised controlled trial of *Helicobacter pylori* eradication in patients on non-steroidal anti-inflammatory drugs: HELP NSAIDs study.

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ABSTRACT: Acid suppression may be more effective than bacterial eradication in healing gastric ulcers caused by nonsteroidal anti-inflammatory drugs (NSAIDs). *Helicobacter pylori* infection in the stomach is strongly associated with ulcers, but NSAIDs may damage the gastric mucous membranes by other physiologic mechanisms. Researchers treated 285 NSAID-associated ulcer patients with omeprazole, an acid-suppressing drug, and either antibiotics or placebo. In six months, about half the patients in both groups were ulcer-free, but more ulcers had healed in the group not given antibiotics.

TEXT:

Summary

Background: The effect of *Helicobacter pylori* in patients receiving non-steroidal anti-inflammatory drugs (NSAIDs) is unclear. We investigated the effects of *H pylori* eradication in patients with current or previous peptic ulceration, dyspepsia, or both who continued to use NSAIDs.

Methods: 285 patients were randomly assigned omeprazole 20 mg, amoxycillin 1000 mg, and clarithromycin 500 mg, twice daily (n=142, *H pylori* eradication treatment), or omeprazole with placebo antibiotics (n=143, controls) for 1 week. All patients received omeprazole 20 mg once daily for 3 weeks until endoscopy, and, if the ulcer was not healed, 40 mg once daily until repeat endoscopy at 8 weeks. Ulcer-free patients with mild dyspepsia continued NSAIDs but not antiulcer treatment. We investigated ulcers with endoscopy at 1, 3, and 6 months and with carbon-13-labelled urea breath test at 3 months.

Findings: The estimated probability of being ulcer-free at 6 months was 0.56 (95% CI 0.47-0.65) on eradication treatment and 0.53 (0.44-0.62) on on control treatment (p=0.80). Time to treatment failure did not differ between groups for ulcers or dyspepsia alone, per-protocol analysis, or final *H pylori* status. 66% (58-74) of the eradication group compared with 14% (8-20) of the control group had a final negative *H pylori* result (p<0.001). Fewer baseline gastric ulcers healed among eradication-treatment patients than among controls (72 vs 100% at 8 weeks, p=0.006).

Interpretation: *H pylori* eradication in long-term users of NSAIDs with past or current peptic ulcer or troublesome dyspepsia led to impaired healing of gastric ulcers and did not affect the rate of peptic ulcers or dyspepsia over 6 months.

Introduction

Helicobacter pylori and non-steroidal anti-inflammatory drugs (NSAIDs) account for nearly all gastroduodenal ulcers and serious ulcer complications.¹⁻⁴ Endoscopic and epidemiological data are conflicting about whether *H pylori* infection increases the risks in NSAID users, has no effect, or is protective.⁵⁻¹⁰

Eradication of *H pylori* substantially decreases the rate of recurrence of gastric and duodenal ulcers in patients not taking NSAIDs,¹¹ but whether this effect occurs in patients on NSAIDs is unclear. In NSAID users, dyspepsia correlates poorly with ulceration but is common in patients infected with *H pylori*.^{12,13}

In a randomised controlled trial we investigated the hypothesis that *H pylori* eradication would decrease dyspepsia in patients taking NSAIDs but would have no effect on ulcer recurrence.

Methods

We did the HELP NSAIDs (*Helicobacter* Eradication for Lesion Prevention with NSAIDs) study in primary-care and secondary-care centres in Hungary, Poland, South Africa, Spain, and the UK. The study was approved by the ethics committees of the participating centres and all patients gave written, informed consent.

Patients

We enrolled men and women aged 18-85 years, who required continuous NSAID treatment and who had *H pylori* infection of the gastric mucosa. Patients were eligible if they had a gastric or duodenal ulcer at the start of the study, had had an endoscopically confirmed ulcer in the previous 5 years, or had moderate or severe NSAID-associated dyspepsia alone or in

combination with ulcers. Patients could be taking any NSAID available for prescription in the participating country (with the exception of phenylbutazone, azapropazone, and ketorolac, which are used for special purposes) for treatment of any musculoskeletal disease at more than a minimum dose (diclofenac 50 mg; ibuprofen 1200 mg; naproxen 500 mg; indometacin 50 mg) on at least 5 days per week. We defined an ulcer as a break of at least 3 mm diameter in the gastric or duodenal mucosa with definite depth.

Our main exclusion criteria were: a previous attempt to eradicate H pylori infection; treatment in the month **before** enrolment with antibiotics, bismuth compounds, or steroids in a dose equivalent to more than 10 mg prednisolone daily; use of drugs, including methotrexate, that could interact with trial drugs; current erosive gastro-oesophageal reflux disease; pyloric stenosis; or substantial bleeding in the upper gastrointestinal tract.

Methods

We asked patients about history of peptic ulcers, likelihood of current ulceration, and current symptoms. If H pylori status was unknown, we did serological testing with **ELISA**. Suitable patients underwent endoscopy and all patients (n=285) who were positive for H pylori infection on helicobacter urease testing¹⁴ (Astra Chemicals, GmbH, Wedel, Germany) entered the study.

We randomly assigned patients, in blocks of eight from a computer-generated list, H pylori eradication therapy with omeprazole 20 mg, amoxycillin 1000 mg, and clarithromycin 500 mg, twice daily (n=142), or control therapy with omeprazole 20 mg twice daily and placebo antibiotics twice daily (n=143), for 1 week. All patients received omeprazole 20 mg once daily until endoscopy at week 4 (figure 1). The study centres were provided with individually sealed code envelopes containing the treatment for each patient. These envelopes were returned to Astra at the end of the study. The code for an individual patient was only to be broken if knowledge of the administered drug was necessary for treatment in an emergency. Patients with duodenal ulcers, gastric ulcers, or both, or moderate or severe dyspepsia in combination with ulcers or alone received omeprazole 40 mg once daily for a further 4 weeks and underwent repeat endoscopy at 8 weeks. We followed up patients who had no ulcers and only mild dyspeptic symptoms at 4-week or 8-week endoscopy for 6 months, **during** which time they continued to take NSAIDs but received no antiulcer treatment. A supply of rescue antacid treatment was available for dyspepsia. We performed endoscopy routinely at 1, 3, and 6 months, and whenever needed.

We took two biopsy samples from the antrum and two from the body of the stomach at the start and end of the study to test H pylori status by the helicobacter urease test. We used also the carbon-13-labelled urea breath test¹⁵ at 3 months of follow-up. We took a rise in the d values at 30 min of more than 5 per mil to be positive for H pylori infection.

We defined H pylori eradication as negative ¹³C-urea breath test at 3 months and negative helicobacter urease at 6 months. If either test was positive, we took the patient to be H pylori positive. If only one test was available, we judged H pylori status on that test alone.

We defined dyspepsia as upper-abdominal pain or discomfort (not relieved by bowel actions or passage of flatus) or nausea. We assessed severity at each visit as none, mild (easily tolerated), moderate (interfering with normal activities), or severe (incapacitating and inability to perform normal activities). To help to identify possible relapses in symptoms, patients kept a daily diary of symptoms in the first month of follow-up and weekly thereafter, and the frequency and severity of symptoms was reviewed with patients at each visit.

Statistical methods

Our primary endpoint was recurrence of ulceration and dyspepsia **after** H pylori eradication. We defined treatment failure as stomach or duodenal ulcers, or both, and 3 or more days of moderate or severe dyspepsia, alone or in combination with ulcers, in any week.

We constructed remission curves from life tables and compared the curves by the logrank test for treatment failure for any reason, because of ulcer recurrence only, or because of dyspepsia only. We did our primary analysis by intention to treat to reflect the pragmatic nature of the study and to avoid confounding by factors, such as compliance, that influence the success of H pylori eradication.¹⁶ We did secondary explanatory analyses of

patients whose treatment was per protocol and analyses relating outcome to final H pylori status.

In the intention-to-treat analysis we included all patients who had at least one dose of medication and who could be assessed. We excluded patients from the per-protocol analysis who did not meet the protocol criteria. We report rates of H pylori eradication primarily for patients who received at least one dose of medication and who were assessed for H pylori status at the end of the trial with the data from helicobacter urease and

13C-urea breath tests. We also report rates for 13C-urea breath test only. We compared eradication of H pylori in the two groups by the Mantel-Haenszel test, stratified by study centre. We used Cox's regression analysis to assess the effect on time to treatment failure of endoscopic classification at entry (dyspepsia, ulcer, both, none), history of peptic ulcer, type of arthritic disease, age, sex, smoking habits, NSAID dose (more than one vs one or less defined daily dose¹⁷), and treatment and steroid use **during** the study.

To calculate sample size, we assumed that the treatment failure rate among controls would be 50% and that a modification in relapse rate by 20% in the eradication group would be clinically meaningful. We estimated that with randomisation of 242 patients, of whom we would be unable to assess 20%, a two-sided log-rank test at the 5% level would have a power of 80%.

Results

Patients

We randomly assigned 285 patients eradication treatment (n=142) or omeprazole plus placebo (n=143, figure 2). Six patients were excluded from the analysis by intention to treat, one because of non-compliance for NSAIDs and five because they did not return for further assessments. 23 patients were excluded from the per-protocol analysis, eight because of non-compliance with study medication, five because they did not meet the entry criteria, one because of a missed visit, and nine because of adverse events, including diarrhoea (two), vomiting (two), and nausea or taste disturbance (two) that prevented adherence to treatment.

Patients in the two groups had similar demographic characteristics (table). Moderate or severe dyspepsia was present in 138 (49%) of all patients (63 eradication, 75 control) at the first visit. 90% of the eradication group and 93% of the control group showed satisfactory compliance with the assigned regimen (defined as intake of at least 85% of the assigned medication).

Efficacy

66% of all patients receiving eradication treatment (95% CI 58-74) were free of H pylori at the end of the study compared with 14% (8-20) of the control group. The highest eradication rates in the participating countries were 78% (South Africa), 73% (UK), 63% (Poland), and 59% (Hungary). Eradication rates by final negative 13C-urea breath test were 86% (80-92) and 22% (14-30) in the eradication and control groups, respectively.

After 4 weeks, 33 (75%) of 44 patients in the eradication group with ulcers at initial endoscopy had no ulcers, compared with 32 (86%) of 37 in the control group. At 8 weeks, 39 (89%) in the eradication group and 37 (100%) in the control group were ulcer-free (figure 3). These differences were attributable to a lower gastric-ulcer healing rate in patients on eradication treatment (50 vs 88% at 4 weeks, 72 vs 100% at 8 weeks, p=0.006). Five patients in the eradication group developed new ulcers (two gastric ulcer, three duodenal ulcer) **during** the treatment period compared with one control (gastric ulcer). One patient in the control group who had a gastric ulcer at baseline and at 8 weeks had developed a new duodenal ulcer at 8 weeks.

In the intention-to-treat analysis, 52 in the eradication group and 58 in the control group reached a treatment failure endpoint. Of these patients, 11 in the eradication group and one in the control group did not become ulcer-free, with or without moderate or severe dyspepsia **during** follow-up. For statistical purposes we took these patients to be day-1 failures. For treatment failure due to all endpoints, the estimated probability of remaining in remission for the 6 months of follow-up was 0.56 (0.47-0.65) for patients on eradication treatment and 0.53 (0.44-0.62) for controls (p=0.80, figure 4).

For patients who developed ulcers, the estimated probability of being in remission at the end of follow-up was 0.80 (0.72-0.88) for the

eradication group and 0.78 (0.76-0.86) for controls. 44% of patients who failed because of gastric ulcers had had gastric ulcers initially. 39% of those who failed because of duodenal ulcers had had duodenal ulcers initially. The estimated probability of being in remission at the end of the study **after** treatment failure because of dyspepsia (without ulcers) was 0.70 and 0.68 for the eradication and control groups, respectively (figure 4).

For patients whose management was per protocol, the estimated probability of remaining in remission was 0.58 (0.49-0.67) for the eradication group and 0.54 (0.45-0.63) for controls. When the data were related to final H pylori status (intention to treat), the estimated probability of remaining in remission was 0.56 (0.47-0.65) for H pylori positive and 0.60 (0.50-0.70) for H pylori negative (figure 4). If final H pylori status was based upon 13C urea breath test alone, the two groups did not differ.

Risk factors for relapse

The only trend close to significance in regression analysis was a decrease in relapse rate in patients who entered the study because of only a past history of ulcers (relative risk 0.67 (0.44-1.01), $p=0.056$). For patients on eradication treatment, the features most associated with increased probability of relapsing were smoking (2.32 (1.31-4.13)) and use of NSAIDs at more than one defined daily dose compared with one or less defined daily dose (2.08 (0.94-4.61)).

The type of lesions found at presentation seemed to be the most important determinant of the likelihood and site of recurrence on treatment failure. 13 (37%) patients with gastric ulcer at enrolment had ulcers at enrolment on treatment failure (six on eradication treatment, seven control); 11 of these were gastric ulcers, one a duodenal ulcer, and in one case gastric and duodenal ulcers. Eight (20%) patients with duodenal ulcers at enrolment had an ulcer at relapse (two on eradication treatment, six control); five were duodenal ulcers, one was a gastric ulcer, and in two cases gastric and duodenal ulcers. Only 19 (9%) patients without ulcers at enrolment developed new ulcers (11 eradication, eight control). Ten of these were gastric ulcers and nine were duodenal ulcers.

Safety

The adverse events most commonly reported were diarrhoea, nausea, and abdominal pain, all reported more commonly for eradication treatment (15%, 5%, and 4%, respectively) than for control treatment (6%, 2%, and 2%). Study drugs were stopped because of adverse events in 16 patients (nine in the eradication group, four while receiving omeprazole 20 mg twice daily, two while receiving omeprazole 20 mg once daily, and one while receiving omeprazole 40 mg daily).

Discussion

Our study was designed to investigate patients who had already experienced gastroduodenal ulceration and drug-associated dyspepsia, since a past history increases the risk of recurrence.² Many of the patients in our study were old and more than 40% had had an endoscopically confirmed ulcer within the previous 5 years. Nearly a third of patients had active ulceration at the start of the study and half had moderate or severe dyspepsia. Our results confirmed previously reported higher rates of recurrence of ulcers, dyspepsia, or both in such patients,^{18,19} but established no benefit from H pylori eradication.

We included in the trial patients with moderate or severe dyspepsia, since this disorder is clinically important in its own right and because H pylori eradication might be useful for drug-related non-ulcer dyspepsia. We have previously reported a strong correlation between the presence of H pylori and NSAID-associated dyspepsia with increased mucosal prostaglandin synthesis, and we formed the hypothesis that prostaglandins might contribute to NSAID-associated dyspepsia in people with H pylori infection.¹² We found, however, that there was no benefit for dyspepsia or ulceration when tested separately by intention to treat.

We would emphasise two important features of the rate of H pylori eradication. First, we did not include metronidazole in the eradication regimen, which was shown in the MACH 1 study²⁰ to be effective in more than 90% of patients, because we tried to obtain the maximum eradication rate and to avoid metronidazole resistance, which is widespread in some of the participating countries. The apparently lower eradication rate seen in our study than in the MACH 1 study (from which NSAID users were excluded) may reflect the stringency of our endpoints (negative results for 13C-urea

breath test and helicobacter urease test). Recognition that single assessments of H pylori, or dual assessments made at related times, may lead to an overestimate of eradication efficacy caused the European Helicobacter pylori Study Group to recommend **before** our study started that eradication be based on two tests at least 2 months apart.²¹ We, however, anticipated such a standard. The eradication rate based only on the 13C-urea breath test was 86%, which is similar to that for the MACH 1 study, in which the 13C-urea breath test was the endpoint.

Other factors that might have influenced eradication in our study include differences in national eradication rates, possibly because of clarithromycin resistance,²² and that NSAID users may differ from other patients in their responsiveness to eradication treatment. This potential difference has not been investigated, but when we analysed the outcome per protocol or by final H pylori status, the recurrence of peptic ulcer or dyspepsia did not differ (in all patients, or only those on eradication treatment). Analysis based on final H pylori status may be biased in favour of patients negative for H pylori since these patients would include those who were more compliant.¹⁶ The lack of difference even with this comparison can, therefore, be taken as evidence against the value of H pylori treatment in the patients studied.

Second, it is unclear why such a high proportion of patients who received control treatment were H pylori negative at the end of the study. Most recrudescence of H pylori infection **after** suppression by omeprazole or antibiotics occurs within 1 month of treatment, but late recrudescence is well recognised. In one large study, recrudescence occurred in 4.1% of patients **after** 3 months,²³ which was the time at which we did breath testing. Whether mucosal changes associated with NSAIDs affect the rate of recrudescence **after** omeprazole is not known. Late recrudescence might contribute to discrepancies between breath test and urease results, which were done at 6 months. A final possibility is that omeprazole interacts with some NSAIDs to kill H pylori. 72% of control-group patients who became H pylori negative were taking diclofenac, which was also the most commonly used NSAIDs in our study. Caselli and colleagues²⁴ found that patients with rheumatoid arthritis taking NSAIDs were less likely to have H pylori infection than controls and have reported that some NSAIDs, including diclofenac, have in-vitro antimicrobial activity against H pylori; other studies have not supported these findings. NSAIDs are, however, weak acids, which probably accumulate in H pylori passively by ion trapping, as they are believed to do in gastric mucosal cells.²⁵ Large changes in pH associated with proton-pump use are, therefore, conceivable, and might influence such trapping or any intrinsic ability of NSAIDs to kill H pylori in vivo. Direct study of the effects of NSAIDs on H pylori at varying pH in vitro and investigation of eradication and recrudescence **after** conventional regimens, as well as **after** omeprazole and diclofenac alone, in NSAID users seems warranted.

Our data contrast with data from Hong Kong.²⁵ That study showed a decrease in rate of peptic (mainly gastric) ulcers over 2 months **after** bismuth-based H pylori eradication in patients on naproxen 250 mg three times daily. The populations of patients in that study and in ours were mutually exclusive. To enter the Hong Kong study, patients had to have no previous or current documented ulcer, not to be taking NSAIDs concurrently, and to have had no more than 1 month's previous exposure at any time to NSAIDs; we positively adopted these entry criteria to study patients at high risk of ulceration or dyspepsia. These two studies have identified different strategies for patients infected with H pylori who are about to start NSAIDs or who are already on NSAIDs and who have experienced previous mucosal injury.

The use of endoscopic assessment of ulceration may be criticised, since prevention of ulcer complications are most important clinically. All antiulcer strategies (whether in NSAID users or other patients) are, however, initially assessed endoscopically and data show that this approach is predictive for the effect on ulcer complications of acid suppression,²⁷ of H pylori eradication in patients not taking NSAIDs,^{28,29} and of misoprostol³⁰ and omeprazole³¹ in patients on NSAIDs. In addition, evidence that H pylori eradication is ineffective compared with omeprazole in the prevention of recurrent ulcer bleeding³¹ gives indirect support to the results of our study.

In this and our previous studies,^{9,10} the site and nature of the initial endoscopic lesion were major determinants of the site and nature of

subsequent relapse and this relation was independent of H pylori status. Our current data reinforce the suggestion that, once substantial mucosal injury has occurred, local mucosal factors have the main influence on relapse and H pylori status becomes largely irrelevant. The Hong Kong study²⁶ raises the possibility that H pylori eradication treatment **before** NSAIDs are started could help to lessen the risks of mucosal injury. Epidemiological and endoscopic data, however, suggest that ulcers and their complications develop frequently in patients on NSAIDs who are H pylori negative^{6,7} and studies lasting longer than 2 months will be needed to establish whether eradication **before** initiation of NSAIDs is of true lasting effectiveness.

Our study does not support existing consensus recommendations^{4,32} to test for and eradicate H pylori in patients already established on NSAIDs who have ulcers or who are believed to be at increased risk of ulcers. These patients are candidates for prophylactic treatment, which, for acid suppression, is more effective in patients who are infected with H pylori than in uninfected patients, paralleling the results with eradication treatment that we report. Direct prospective study of the interaction between eradication and maintenance treatments and the possibility that new and established NSAID users may differ in their responses to eradication, is needed.

Trial participants

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Contributors

C J Hawkey was responsible for protocol design, recruitment patients, input to the statistical analysis, and the writing of paper. Z Tullasay was a member of the steering committee, contributed to protocol design, and was involved in recruitment of patients and the editing of paper.

L Szczepanski, C J van Rensburg, and A Filipowicz-Sosnowska enrolled patients into the study and were involved in the writing and editing of the paper. A Lanas was a member of the steering committee and contributed to protocol design, recruitment of patients, and the editing of paper. C M Wason was the study coordinator, collected data, and was involved in the writing and editing of the paper. R A Peacock did the statistical analysis and was involved in the writing and editing of the paper. K R W Gillon was involved in protocol design, study coordination, and had input into statistical analysis, and was responsible for the writing of the paper.

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Randomised trial of eradication of *Helicobacter pylori* before non-steroidal anti-inflammatory drug therapy to prevent peptic ulcers.

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ABSTRACT: The risk for stomach ulcers among patients infected with *Helicobacter* (*H.*) *pylori* may be reduced if the infection is treated **before** the patient ever takes any non-steroidal anti-inflammatory drugs (NSAIDs). The stomach ulcer rate was evaluated **after** 92 infected patients never exposed to NSAIDs were treated with either eight weeks of naproxen alone or eight weeks of a triple therapy followed by naproxen. Only 3% of the patients successfully treated for the *H. pylori* infection developed ulcers while 26% of those with continuing infections developed ulcers. Triple therapy eliminated significantly more infections than naproxen alone.

TEXT:

Summary

Background *Helicobacter pylori* infection is common in patients with peptic ulcers caused by the use of non-steroidal anti-inflammatory drugs (NSAIDs). But the pathogenic role of *H. pylori* in this disease is controversial. We studied the efficacy of eradication of *H. pylori* in the prevention of NSAID-induced peptic ulcers.

Methods We recruited patients with musculoskeletal pain who required NSAID treatment. None of the patients had previous exposure to NSAID therapy. Patients who had

H. pylori infection but no pre-existing ulcers on endoscopy were randomly allocated to naproxen alone (750 mg daily) for 8 weeks or a 1-week course of triple therapy (bismuth subcitrate 120 mg, tetracycline 500 mg, metronidazole 400 mg, each given orally four times daily) **before** administration of naproxen (750 mg daily). Endoscopy was repeated **after** 8 weeks of naproxen treatment or when naproxen treatment was stopped early because of bleeding or intractable dyspepsia. All endoscopic examinations were done by one endoscopist who was unaware of treatment assignment. The primary endpoint was the cumulative rate of gastric and duodenal ulcers.

Findings 202 patients underwent endoscopic screening for enrolment in the trial, and 100 eligible patients were randomly assigned treatment. 92 patients completed the trial (47 in the naproxen group, 45 in the triple-therapy group). At 8 weeks, *H. pylori* had been eradicated from no patients in the naproxen group and 40 (89%) in the triple-therapy group ($p < 0.001$). 12 (26%) naproxen-group patients developed ulcers: five had ulcer pain and one developed ulcer bleeding. Only three (7%) patients on triple therapy had ulcers, and two of these patients had failure of *H. pylori* eradication ($p = 0.01$). Thus, 12 (26%) patients with persistent *H. pylori* infection but only one (3%) with successful *H. pylori* eradication developed ulcers with naproxen ($p = 0.002$).

Interpretation Eradication of *H. pylori* **before** NSAID therapy reduces the occurrence of NSAID-induced peptic ulcers.

Introduction

Peptic ulcer disease caused by non-steroidal anti-inflammatory drug (NSAID) therapy is a health-care issue worldwide. In the UK, about 15 million people aged over 60 years take NSAIDs at any one time.¹ Patients who take NSAIDs have a four-fold to six-fold increased risk of developing peptic ulcers.^{2,3} Every year, about 12 000 ulcer complications occur in the UK as a result of NSAID therapy.⁴ Although chronic NSAID users are at increased risk of ulcer disease, those who take intermittent short-course NSAID therapy are also at risk of the disease. Most ulcer complications tend to develop in the first few weeks of therapy, particularly among individuals without previous exposure to NSAIDs.^{3,5} The risk of ulcer complications is greatest among the elderly and patients with comorbid disease.^{2,3} Up to 60% of ulcer complications occur without antecedent symptoms.⁶ The annual direct medical costs associated with these complications is about \$39 billion in the USA.⁷

Previous studies have shown that prophylactic therapy with misoprostol, famotidine, and omeprazole is effective in the prevention of NSAID-induced ulcers.⁸⁻¹⁰ However, these prophylactic regimens are very expensive.¹¹ Cost-effective measures to prevent NSAID-induced ulcer disease are not available.

H. pylori is present in about 50% of patients with NSAID-associated ulcer disease.^{12,13} Studies of the interaction between *H. pylori* infection and NSAIDs have reported conflicting findings.¹²⁻²⁶ Whether *H. pylori* infection increases the risk of ulcer development in users of NSAIDs is not known.

The aim of our study was to investigate whether eradication of *H.*

pylori **before** the start of NSAID therapy reduced the occurrence of gastroduodenal ulcers in patients without previous exposure to NSAIDs. We postulated that underlying infection with *H pylori* increases the predisposition of NSAID users to develop ulcers. Thus, eradication of *H pylori* could protect these patients from developing peptic ulcers.

Methods

Patients with musculoskeletal disorders that required NSAID therapy were eligible for the study. We recruited patients from the medical outpatient clinic at Prince of Wales Hospital, Hong Kong, and from the family clinic of the Chinese University of Hong Kong. We excluded patients if they: were younger than 18 years; had been previously exposed to NSAIDs (including aspirin) for longer than 1 month; had taken NSAIDs (including aspirin), antiulcer drugs, steroid, anticoagulants, or cytotoxic agents in the previous 8 weeks; had received antihelicobacter therapy; had a history of peptic ulcer disease or gastric surgery; or if they had renal impairment (serum creatinine >200 mmol/L). Eligible patients were invited to take part in the study and were given a detailed explanation. Those who gave their informed written consent to take part in the study underwent upper-gastrointestinal endoscopy. The study protocol was approved by the ethics committee of the Chinese University of Hong Kong.

All endoscopic assessment was done by a one endoscopist (FKLC) to eliminate between-observer variation. We defined a peptic ulcer as a circumscribed mucosal break 5 mm or more in diameter with a well-defined ulcer crater, whereas smaller or superficial lesions were classified as erosions. Ulcer size was measured by standard Olympus biopsy forceps (Olympus FG-25K), with the fully open instrument equivalent to 5 mm. We excluded patients with ulcers detected at baseline endoscopy.

Five random biopsy specimens were taken from the antrum; two specimens were used for the rapid urease test (CLO test, Delta West, Bentley, Western Australia) and three samples were sent for histological assessment with haematoxylin and eosin stain and Warthin-Starry stain. The histological assessment was done by one pathologist (KFT) who was unaware of treatment assignment. A patient was judged to be infected with *H pylori* if they had a positive rapid urease test that was confirmed by the histology. A positive rapid urease test alone was not deemed sufficient for the diagnosis of *H pylori* infection.

Patients who were confirmed to have *H pylori* infection and did not have an ulcer at baseline endoscopy were randomly assigned to one of two treatment groups. We used a list of computer-generated random numbers for treatment assignment. Patients were assigned naproxen 750 mg daily in three divided doses at 8 h intervals for 8 weeks, or a 1-week course of triple therapy (bis-muth subcitrate 120 mg, tetracycline 500 mg, and metronidazole 400 mg, each given orally four times daily) followed by naproxen 750 mg daily in three divided doses at 8 h intervals for 8 weeks. Dologesic (propoxyphene napsylate 50 mg, paracetamol 325 mg) was given to patients for temporary pain relief **during** triple therapy, or as an adjuvant therapy to naproxen if requested by patients.

A research nurse interviewed each patient about their smoking habits, alcohol consumption, drug history, and concurrent medical illness. Complete blood count and renal function tests were carried out. We assessed drug compliance by counting unused tablets. Endoscopy was repeated by the same endoscopist **after** 8 weeks of naproxen therapy to document gastroduodenal ulcers. *H pylori* status was reassessed by repeating antral biopsies. We defined eradication of *H pylori* as negative rapid urease test and absence of the bacteria on histology. The endoscopist and pathologist were unaware of the treatment allocation and previous endoscopic or histological findings. Those patients who developed intractable dyspeptic symptoms that required the early termination of naproxen, or who had gastrointestinal haemorrhage (defined as haematemesis or melaena) underwent endoscopy **before** 8 weeks.

The primary endpoint was the cumulative rate of gastric and duodenal ulcers. All patients who returned for follow-up endoscopy were included in the intention-to-treat analysis. We did a per-protocol analysis for those patients who took more than 60% of the prescribed naproxen, or at least 5 days of the triple therapy in patients on antihelicobacter treatment. We also compared the clinical outcome of patients with or without successful eradication of *H pylori* infection.

We estimated the sample size based on our previous study, which showed an ulcer prevalence of 33% in NSAID users who were infected with *H pylori*

com- pared with 6% in NSAID users who did not have H pylori infection.¹⁹ To achieve a statistical power of 85%, with an a error of 5%, 45 patients were needed in each treatment group. Under the assumption of a drop-out rate of 10% in each group, we calculated that 100 patients were required in the study. The results of the two treatment groups were compared by two-tailed Fisher's exact test, Pearson χ^2 test, and Mann-Whitney U test. A p value less than 0.05 was taken as significant.

Results

202 consecutive patients agreed to take part and underwent endoscopy for enrolment in the trial. Of these patients, 91 were not infected by H pylori, two had ulcers at initial endoscopy, and nine patients with H pylori refused to participate in the study **after** endoscopy. 100 patients were randomly assigned treatment in two groups of 50. Eight withdrew **after** randomisation and defaulted follow-up (naproxen group: one refused to undergo second endoscopy, one refused to take part, and one patient had abdominal pain **after** taking naproxen; triple therapy plus naproxen group: adequate pain relief with dologesic in two, one refused to take part, one patient with palpitation **after** taking naproxen, and one patient with intolerance to triple therapy). 92 patients completed the study: 47 in the naproxen group and 45 in the triple-therapy group.

The flow of patients is shown in the trial profile. The two groups were well matched for age, sex, smoking and drinking habits, and underlying arthritis (table 1). In the triple-therapy group, there were more patients with comorbid illness and gastric erosions were more frequent on baseline endoscopy than in the naproxen group, but the difference was not significant. 41 patients completed at least 5 days of the prescribed 1-week triple therapy.

40 (89%) triple-therapy patients had successful eradication of H pylori, versus none in the naproxen group ($p < 0.001$).

The cumulative rate of gastric and duodenal ulcers **after** 8 weeks of treatment is shown in table 2. In the intention-to-treat analysis, 12 (26%) patients in the naproxen group compared with three (7%) in the triple-therapy group developed peptic ulcers ($p = 0.01$). The size of ulcers ranged from 5 mm to 10 mm (mean diameter of 6 mm). Seven patients had more than one ulcer; one patient had both gastric and duodenal ulcers. In the naproxen group, six of the 12 patients developed symptomatic ulcers that required early termination of naproxen: five had intractable dyspepsia (two developed ulcers **after** 2 weeks of naproxen, three had ulcers at 5-7 weeks), and one had ulcer bleeding **during** week 7. Of the three patients who developed ulcers in the triple-therapy group, two had failure of H pylori eradication (one completed triple therapy but developed intractable dyspepsia and an ulcer was detected **after** 1 week of naproxen, and the other patient took triple therapy for only 2 days because of gastrointestinal upset and an ulcer was found on follow-up endoscopy at 8 weeks). Even when we assumed that all defaulters (except those who responded adequately to dologesic alone) were treatment failures, pretreatment with antihelicobacter therapy still significantly reduced the rate of ulcers: 15 patients with ulcers in the naproxen group versus six in the triple-therapy group ($p = 0.048$). One patient in each group had epigastric pain **before** 8 weeks, but endoscopy did not reveal any ulcers. Other endoscopic lesions were also detected (subepithelial haemorrhage and erosions), but there was no significant between-group differences.

Comparison of the rate of ulcers in patients with and without successful eradication of H pylori, showed that only one (25%) patient developed ulcer **after** successful eradication, whereas 12 (26%) patients infected with H pylori developed ulcers with naproxen ($p = 0.002$). Of the 15 patients who developed peptic ulcers in both treatment groups, 11 (73%) were older than 60 years (range 49-84 years). Comorbid disease was present in 11 (73%) patients (seven had more than one disease).

The per- protocol analysis included 43 naproxen-group patients and 38 triple-therapy patients (table 3). Four patients in the naproxen group took less than 60% of the drug prescribed: one had adequate pain relief with dologesic alone, one did not have adequate pain relief with naproxen, and two had dyspeptic symptoms that disappeared when the frequency of naproxen intake was reduced. In the triple-therapy group, three patients took the antibiotics for less than 5 days because of gastrointestinal upset, and four did not take naproxen regularly (two had adequate pain relief with dologesic alone, one found naproxen ineffective, and one complained of leg cramps and blurred vision **after** taking naproxen). These patients were

excluded from the analysis. The cumulative rate of gastric ulcers was significantly lower in patients pretreated with triple therapy (p=004).

Discussion

The interaction between H pylori infection and use of NSAIDs in the pathogenesis of peptic ulcer is unclear. Most of the available data is from cross-sectional studies based on chronic NSAID users, with conflicting results.¹²⁻²⁰ Two studies reported a higher rate of H pylori in NSAID users with gastroduodenal lesions than in those with normal mucosa.^{13,20} Others found significantly more ulcers in NSAID users who were H pylori positive than in users not infected with H pylori.^{12,16,19} But these findings were not confirmed by other investigators.^{14,15,17,18} Such conflicting findings arise from different study designs and outcome measures--for example, they either assessed the rate of H pylori in NSAID users with or without mucosal damage^{13,14} or, conversely, the rate of mucosal damage in NSAID users with and without H pylori.¹⁵⁻¹⁹ Thus, direct comparison of results is difficult. These cross-sectional studies do not, therefore, provide definite evidence for or against a link between H pylori and NSAIDs in the development of peptic ulcers. Most of the few published prospective trials did not indicate that H pylori is a risk factor for NSAID-induced gastroduodenal damage.²¹⁻²⁵ Three studies reported that H pylori did not affect the severity of gastroduodenal damage **after** short-term administration of NSAIDs.²¹⁻²³ However, the data were mostly derived from studies that involved small numbers of young healthy volunteers. Any interaction between H pylori and NSAIDs could have been obscured by the small number of individuals included with low event rates in low-risk groups. Two long-term longitudinal studies of chronic NSAID users gave conflicting results.^{25,26} Kim and colleagues²⁵ reported no significant increase in the rate of gastroduodenal ulcers among chronic NSAID users with H pylori infection; patients with erosions detected at baseline were excluded in the study. By contrast, Taha and colleagues²⁶ found that patients with H pylori-positive duodenal erosions were more likely than patients who were not infected with H pylori to develop ulcers **during** NSAID treatment. Selective recruitment of chronic users without complications may introduce bias, since those susceptible individuals could have dropped out early because of NSAID intolerance or complications.^{1,3}

The best evidence of a positive interaction between H pylori and NSAIDs in the pathogenesis of ulcer would be obtained by investigating the effects of H pylori eradication on the occurrence of NSAID-induced ulcers. Seppala and co-workers²⁷ observed that gastric ulcer relapse seemed to be reduced in a small group of NSAID users **after** eradication of H pylori. A prospective study by Bianchi and colleagues²⁸ assessed the efficacy of amoxicillin-omeprazole dual therapy on the healing and recurrence of ulcers in chronic NSAID users who were infected with H pylori. They found no significant benefit although there was a numerical trend towards a higher rate of ulcer recurrence in patients with H pylori infection. However, the rate of H pylori eradication was very low, and their result was further limited by the small number of patients with ulcer recurrence and the use of different NSAIDs with variable ulcerogenic potentials.

Our present study is the first prospective randomised trial to look at whether eradication of H pylori **before** the start of NSAID therapy reduces the subsequent risk of ulcer formation. Our findings establish a pathogenetic role for H pylori in the development of NSAID-induced ulcers, in that prophylactic eradication of H pylori significantly reduced the 8-week cumulative rate of NSAID ulcers by almost four-fold. The degree of protection conferred by eradication of H pylori was similar to that provided by acid-suppression drugs.^{9,10} The rate of ulcers in relation to H pylori status was consistent with our previous findings¹⁹ and those reported by Ekstrom and co-workers.¹⁰ Since the risk of ulcer is high **during** the first weeks of NSAID therapy, particularly in those without previous exposure,^{3,5} our finding is relevant to patients who need intermittent treatment for exacerbation of such disorders as osteoarthritis.

Our study differs from others in that most patients in our series were elderly with concomitant illness--ie, at high risk of developing NSAID-induced ulcer complications. We did not recruit patients who were already on long-term NSAIDs, or those with frequent previous exposure to NSAIDs, to avoid any potential selection bias.^{3,5} All patients received the same dosage of naproxen to eliminate any confounding effects associated with different doses and types of NSAIDs.³ In addition, all endoscopic

examinations were done by one endos- copist who was unaware of treatment allocation to eliminate bias and between-observer variation. The lower rate of ulcers in patients on antihelicobacter therapy might be related to the antiulcer effect of bismuth, but because bismuth was given for only 1 week **before** the start of NSAID therapy, this effect was kept to a minimum.

Our results show that NSAID-induced ulceration can be reduced by eradication of *H pylori* **before** NSAID administration and establish *H pylori* infection as a risk factor for NSAID-induced ulcer disease. Coprescription of antiulcer prophylactic drugs can reduce the rate of NSAID ulcers, but the high cost of such treatment prevents its widespread use. Although it would be costly and impractical to carry out endoscopy on every patient **before** administration of NSAID, *H pylori* status can be easily determined by an office-based serology test. Determination of *H pylori* and eradication in infected patients should be recommended **before** the start of NSAID therapy.

Contributors

Francis K L Chan, Joseph J Y Sung, S C Sydney Chung, and Jean Woo were responsible for the original design of the study. Francis K L Chan, Y T Lee, Vincent K S Leung, and Cynthia S Y Chan coordinated the study. Data collec- tion, statistical analysis, and independent outcome assessment were conducted by Francis K L Chan, K F To, M Y Yung, and Vincent K S Leung. The manuscript was edited by Joseph J Y Sung, S C Sydney Chung, Edmund K M Li, and Jean Woo. All the authors contributed to the execution of the study and the writing of the paper.

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Effect of Helicobacter pylori eradication on G-cell and D-cell density in children.

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ABSTRACT: Children with *Helicobacter* (H.) *pylori* infection may have lower concentrations of antral somatostatin, a hormone that inhibits synthesis and release of the enzyme gastrin, and is associated with duodenal ulcer. Previous studies have focused on antral somatostatin and H. *pylori* infection in adults but not in children. Hypergastrinemia may occur in children with H. *pylori* because of the low amount of antral somatostatin, which inhibits antral gastrin cells, or G cells, from secreting gastric acid. When H. *pylori* was treated, G cell concentrations decreased and the density of cells immunoreactive for somatostatin, or D cells, increased. D-cell density and antral somatostatin concentration may be linked to duodenal ulcer but only with H. *pylori* infection present. Results may not be applicable to children from developed countries; the study was done on children in Brazil, a developing country.

AUTHOR ABSTRACT: *Helicobacter pylori* infection is associated with abnormalities in serum gastrin concentration, antral gastrin and somatostatin content, and D-cell density in adults. We have studied the effects of H *pylori* infection in children. We studied 13 children positive for H *pylori* and 7 negative children. The median antral somatostatin content was significantly lower in the positive than in the negative group (0.69 [range 0.35-0.91] vs 1.31 [0.73-1.67] ng/mg, $p = 0.007$). Both antral and serum gastrin concentrations were significantly higher in the positive group (30.1 [15.3-83.6] vs 14.8 [13.8-28.8] ng/mg, $p = 0.008$; and 89.9 [59.4-313.2] vs 29.5 [13.9-71.1] pg/mL, $p = 0.006$). Treatment to eradicate H *pylori* was successful in 11 of the 13 positive patients. With eradication antral somatostatin increased to within the normal range (by a median of 0.41 [0.21-0.86] ng/mg to 1.10 [0.81-1.55] ng/mg, $p = 0.016$). Serum and antral gastrin decreased (by 37.1 [5.5-265.2] pg/mL to 52.8 [21.4-267.5] ng/mg, $p = 0.001$; and by 8.0 [2.0-47.2] ng/mg to 22.1 [10.9-37.5] ng/mg, $p = 0.001$). Eradication of H *pylori* also significantly increased antral D-cell density (8 [5-22] to 15 [9-22] cells per mm, $p = 0.031$) and decreased G-cell density (138 [89-161] to 88 [33-121] cells per mm, $p = 0.016$). The hypergastrinaemia in children positive for H *pylori* may be due to a deficiency of antral somatostatin, which inhibits gastrin synthesis and release.

TEXT:

Introduction

Circulating gastrin concentrations are higher in patients positive for *Helicobacter pylori* than in subjects without the bacterium.[1,2] Moreover, clearance of the microorganism with antimicrobial agents leads to a rapid decrease in serum gastrin concentration among patients with and without duodenal ulcer.[3-5]

Since a delicate balance of hormonal mechanisms regulates the secretion of gastrin,[6] the hypergastrinaemia might be due to a decrease in somatostatin, a powerful and effective inhibitor of antral gastrin cells (G cells) and gastric-acid secretion.[7,8] Patients positive for H *pylori* have lower antral somatostatin concentrations than negative individuals[9] and eradication of H *pylori* in patients with duodenal ulcer is followed by increases in the antrum of mRNA for somatostatin or somatostatin itself and in density of cells immunoreactive for somatostatin (D cells).[10,11] However, there have been no previous studies on this subject in the children. Furthermore, the effect of H *pylori* eradication on G cells and D cells and on antral concentrations of somatostatin and gastrin has not been assessed previously in H *pylori* positive subjects without duodenal ulcer.

We have measured antral gastrin and somatostatin concentrations in H *pylori* positive and negative children and studied the effect of eradication of the microorganism on the concentration of these gastric regulatory peptides and their cells of origin.

Patients and methods

This study was approved by the ethics committee of the Hospital das Clinicas/UFGM. Informed consent to take part was obtained from all children and their parents.

We studied 20 children (6 boys, 14 girls; mean age 9.9 [range 6-14] years) who underwent endoscopy for investigation of upper abdominal pain and who did not have a history of smoking or underlying disorders. None had received non-steroidal anti-inflammatory drugs, histamine H₂-receptor antagonists, or any other medication for at least 30 days **before** the study.

13 patients (4 boys, 9 girls; mean age 9.8 [6-14] years) were positive for H pylori and 7 (2 boys, 5 girls; mean age 9.9 [7-12] years) were negative for the bacterium by culture, urease test, and carbolfuchsin-stained smear.[12,13] Positive children received a triple antimicrobial therapy schedule with furazolidone 6-8 mg/kg daily up to 300 mg, amoxycillin 50 mg/kg daily up to 750 mg, and metronidazole 20-30 mg/kg daily up to 750 mg in three daily doses **after** meals, for 7 days. These patients were restudied 3 months **after** treatment stopped.

Three tissue samples for somatostatin and gastrin assays were taken from the lesser curvature of the antrum, immediately frozen, and stored in liquid nitrogen until the time for processing. Hormones were extracted[14] and immediately frozen and lyophilised.

Tissue somatostatin concentration was measured by radioimmunoassay (Royal Postgraduate Medical School, London, UK) by a slight modification of Williams et al's method.[11,15] Gastrin was assayed by a double- **antibody** iodine-125 radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). This assay system uses an **antibody** that can detect active gastrins (G-17 and G-34), has very low cross-reactivity with other naturally occurring compounds, and has a detection limit of about 12 pg/mL. The assays were done **during** a single session **after** all samples had been collected.

For measurement of the cell density of cells immunoreactive for somatostatin and gastrin (D cells and G cells) 3 to 4 biopsy samples from the lesser curvature of the antrum, 1-2 cm from the pylorus, were immediately fixed in Bouin's solution for 18 h, dehydrated, and embedded in paraffin. 4 [μm] thick sections cut perpendicular to the surface of the mucosa were deparaffinised and immunoreactive cells were identified on serial sections by the immunoperoxidase reaction[16] (gastrin and somatostatin **antibodies** from Dr J M Polak, London, UK). We examined only sections showing the whole area between surface and muscularis mucosae and with an intact mucosa. At least 10 adjacent areas encompassing the mid-zone of the mucosa from 3 or 4 different sections were used for counting of G and D cells on a microscope with x 10 ocular and a x 40 objective. The cells were counted independently by two observers and the mean number of cells per mm of muscularis mucosae was calculated. 4 [μm] sections were also stained with haematoxylin and eosin for histology.

The Wilcoxon signed-rank test for paired samples and Mann-Whitney test for unpaired data were used in statistical analysis. Differences were taken as significant when $p < 0.05$.

Results

Serum gastrin concentration was significantly higher in the 13 patients positive for H pylori than in the 7 negative subjects (median 89.9 [range 59.4-313.2] vs 29.5 [13.9-71.1] pg/mL; $p = 0.006$). There were also significant differences between positive and negative children in antral gastrin content (30.1 [15.3-83.6] vs 14.8 [13.8-28.8] ng/mg; figure 1; $p = 0.008$). Antral somatostatin content was measured in only 7 of the 13 children positive for H pylori; nevertheless it was significantly lower than in the negative children (0.69 [0.35-0.91] vs 1.31 [0.73-1.67] ng/mg; $p = 0.007$).

On histology all children positive for H pylori had antral gastritis, which was active (polymorphonuclear neutrophils present in the lamina propria, glandular and superficial epithelium, or both) in 12, but no atrophy or intestinal metaplasia was observed.

In 11 of 13 children who received triple antimicrobial therapy, H pylori was eradicated, as confirmed by a negative urease test, culture, and carbolfuchsin-stained smears. Antral gastrin content was measured **before** and **after** eradication of H pylori in all 13 but antral somatostatin content and D-cell and G-cell densities were measured in only 7 patients, because the biopsy samples were not adequate or available in the other cases.

With H pylori eradication the antral mucosa somatostatin content increased significantly ($p = 0.016$) by a median of 0.41 (range 0.21-0.86 ng/mg) to 1.10 (0.81-1.55) ng/mg and the antral gastrin content decreased

significantly ($p = 0.001$) by 8.0 (2.0-47.2) ng/mg to 22.1 (10.9-37.5) ng/mg (figure 2). These changes in antral somatostatin and gastrin meant the post-eradication concentrations were similar to the baseline concentrations in the H-pylori-negative group ($p = 0.6$ and $p = 0.5$, respectively). There was also a significant fall ($p = 0.001$) in serum gastrin concentration **after** eradication of the microorganism (fall of 37.1 [5.5-265.2] pg/mL to 52.8 [21.4-267.5] pg/mL).

Eradication of H pylori was accompanied by a significant decrease in G cells from a median of 138 (89-161) cells per mm to 88 (33-121) cells per mm ($p = 0.016$) and by an increase in D-cell density from a median of 8 (5-22) cells per mm to 15 (9-22) cells per mm ($p = 0.031$) (figure 3).

Discussion

Patients positive for H pylori have abnormalities in the regulation of gastrin release that may be the link between H pylori and duodenal ulcer, since gastrin could have an important role in acid hypersecretion. Gastrin-stimulated acid secretion is increased in patients positive for H pylori with or without duodenal ulcer, but more so in duodenal ulcer patients. Eradication of H pylori leads to a reduction in gastrin-mediated acid secretion. [17]

The mechanism by which H pylori changes gastrin metabolism, however, is only partly elucidated. The changes may be caused by cytokines, interleukin-2 and interferon, released by the inflammatory cells of the antral mucosa, which can stimulate G cells. [18] The changes could also result from a decrease in antral somatostatin, which inhibits gastrin synthesis and release. [11,19]

In this study we showed that changes in D-cell density and antral somatostatin content are a characteristic not of duodenal ulcer patients per se, but of H pylori infection. Possible explanations of how H pylori infection reduces the number of antral D cells and antral somatostatin include: alkalinisation of the microenvironment as a result of ammonia production from urea by H pylori urease, other noxious metabolic products of the bacterium, or inflammation of the antral mucosa induced by the infection that may inhibit antral D cells. Some studies have found no direct effect of high antral surface pH due to the action of H pylori urease in hypergastrinaemia. [19,20] In those studies, however, serum gastrin was measured as early as 24h **after** the inhibition of H pylori urease activity by acetohydroxamic acid or antibiotics. [19,20] It is likely that chronic exposure to ammonia lowers the number of antral D cells and there could be a delay between H pylori eradication, increased D-cell density, and consequently decreased G-cell function.

Our results show some similarities and some differences between these children and adults with H pylori infection we studied previously. [11] Antral somatostatin content and the number of D cells increased and the concentration of gastrin decreased significantly **after** eradication of the bacterium in both groups. However, we observed a decrease in G-cell density **after** H pylori eradication in children. In adults, the high serum and antral gastrin concentrations found **before** H pylori eradication have been interpreted as showing enhanced functional activity of G cells rather than an increase in G cell mass, since no differences in number of G cells have been found [10,11,21] between patients positive and negative for H pylori or **after** eradication of the microorganism. Nakanishi et al, [22] however, described an adult with H pylori, duodenal ulcer, and primary gastrin-cell hyperplasia in whom eradication of the microorganism was accompanied by a return to normal serum and antral gastrin concentrations and a significant decrease in gastrin cell density. [22] Thus, some adults with H pylori could also present G-cell hyperplasia.

Although we observed a decrease in antral G-cell density **after** H pylori eradication, we cannot conclude that this is the case in all children with the infection. The pattern of gastritis differs between children from developing and developed countries. For example, we observed gastritis with inflammatory activity in 12 of 13 children positive for H pylori, a result similar to that obtained for Hong Kong children (96%), [23] but much higher than the 35-45% of affected children in developed countries. [24,25] Time of infection (duration of gastritis) is probably longer in children from developing countries, who may well acquire the infection earlier; this difference could also contribute to the G-cell hyperplasia observed in our study. Further studies are necessary to explain the mechanisms involved and to clarify the differences observed between children and adults.

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SPECIAL FEATURES: illustration; graph

DESCRIPTORS: *Helicobacter* infections--Complications; Somatostatin--

Physiological aspects; Duodenal ulcer--Causes of; Gastrin--Physiological aspects

3/9/45 (Item 6 from file: 149)

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Duodenal ulcer healing by eradication of Helicobacter pylori without anti-acid treatment: randomised controlled trial.

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ABSTRACT: Patients with duodenal ulcers and Helicobacter (H.) pylori infection may be healed with a one-week regimen consisting of three drugs, though pain is reduced more rapidly with the addition of a fourth drug. Researchers assigned 160 patients to take either bismuth subcitrate, tetracycline and metronidazole (BTM) for one week or BTM for one week plus omeprazole for four weeks. Of 132 patients who completed the four-week study, 60 of 65 patients who took BTM and 63 of 67 patients who took BTM plus omeprazole had healed ulcers. H. pylori infections were cured among 61 (94%) patients receiving BTM and 66 (98%) of those receiving BTM plus omeprazole. **During** the first week of therapy, symptoms were relieved more effectively among patients taking omeprazole in addition to BTM. These patients averaged about three days of ulcer pain, while those not taking omeprazole averaged about four days of pain.

AUTHOR ABSTRACT: Randomised trials have shown that duodenal ulcers treated by [H.sub.2] blockers heal faster if Helicobacter pylori is eradicated concurrently. It remains unknown whether eradication of H pylori without suppression of acid-secretion, is sufficient to allow healing.

153 patients with H pylori infection and duodenal ulcer were randomised to receive either a 1-week course of bismuth subcitrate, tetracycline, and metronidazole (76), or omeprazole for 4 weeks with the same three-drug regimen for the first week (77). Endoscopy and antral biopsies were done at entry and 4 weeks **after** treatment. 132 patients were suitable for analysis. Duodenal ulcers healed in 60 (92%; 95% CI 86-100%) patients taking bismuth, tetracycline, and metronidazole compared with 63 (95%; 88-100%) taking omeprazole in addition to the three other drugs. H pylori was eradicated in 61 (94%; 88-100%) who received only three drugs compared with 66 (98%; 96-100%) who received omeprazole as well. Symptoms were reduced more effectively **during** the first week in patients who received omeprazole (p = 0.003).

We conclude that a 1-week regimen of bismuth, tetracycline, and metronidazole for patients with H pylori and duodenal ulcer eradicates the organism and heals the ulcer in most patients. Concurrent administration of omeprazole reduces ulcer pain more rapidly but has no effect on ulcer healing.

TEXT:

Introduction

For most of the last 70 years, methods of healing duodenal ulcers have relied on reduction of gastric acid. Recently, other factors including mucosal prostaglandins, pepsin, and the mucous layer have been implicated in duodenal ulcer and the pharmacodynamics of drugs such as misoprostil[1] confirm this. The link between Helicobacter pylori and duodenal ulcer indicates another - possibly the most important - factor in the causes of this disease. Several randomised trials[2-4] have shown that provided H pylori is eradicated, the relapse rate of duodenal ulcer is reduced from around 85% to 5-10% in the following year. Studies have shown that duodenal ulcers treated with [H.sub.2] blockers heal faster if H pylori is eradicated concurrently.[5,6] It remains unknown whether eradication of H pylori alone, without acid suppression, is sufficient to heal duodenal ulcers. If so, this would provide further evidence of a causal link between

H pylori and duodenal ulcer. We did a randomised trial to test this hypothesis.

Patients and methods

All patients coming to the Prince of Wales Hospital between Nov 1991, and Sept 1992, with dyspepsia and found to have an endoscopically proven duodenal ulcer, were entered into our trial unless they were aged under 16 or over 75 years, had a history of gastrointestinal bleeding within the previous 4 weeks, had had previous acid-reduction surgery, were pregnant, or intended to leave Hong Kong within 5 weeks of starting treatment. Eligible patients had antral biopsies for urease activity (CLO test, Delta West, **Western** Australia), microscopy, and culture to detect H pylori. Patients were considered to be H pylori positive or H pylori negative based on the results of culture. All positive cultures were tested for sensitivity to tetracycline and metronidazole. Our laboratory techniques have been published elsewhere.[7] Patients were randomised by instructions in sealed envelopes to receive either a 1-week course of bismuth subcitrate 120 mg, tetracycline 500 mg, and metronidazole 400 mg (BTM) four times per day, or the same regimen for 1 week plus omeprazole 20 mg per day for 4 weeks (OBTM); all treatments starting on the day of randomisation. The average duration and severity of each patient's symptoms **during** the week **before** first endoscopy were recorded. Each patient was then given a diary card to record symptoms **during** the next 5 weeks. **After** 1 week of treatment, all patients were interviewed by a research nurse to check on drug compliance, side effects, and ulcer symptoms. Patients randomised to receive 1 week's treatment of BTM alone were then given 60 antacid tablets (neutralising capacity 11.5 mmol acid per tablet) to be taken as required. Delaying antacid ingestion until **after** the first week was to prevent possible interaction between these and the antibiotics.

5 weeks **after** trial entry (4 weeks **after** stopping anti-H pylori treatment), patients returned their diary cards and remaining tablets, and underwent further endoscopy and tests as described above. The endoscopist and microbiologist were unaware of the patient's randomisation. Patients gave informed consent and the trial was approved by the Prince of Wales Hospital ethical committee.

Statistics

Trial size was calculated by assuming a healing rate of 95% for patients given OBMT. 150 patients were required to show with a power of 99% that healing rates were within 10% of each other. Allowing for dropouts and exclusions, 160 patients were randomised. Comparability between the two groups of patients was tested by [X.sup.2] analysis, symptoms were compared by Mann-Whitney U test, and results of ulcer healing were analysed by Fisher's exact test.

Results

160 patients with duodenal ulcer were randomised (table 1). The two groups were well matched with respect to age and sex. Pretreatment symptoms were worse in the group of patients who took omeprazole and there were more smokers in this group but neither difference was significant ($p = 0.06$ and 0.11 , respectively). No patients were taking nonsteroidal anti-inflammatory drugs, none had received anti-H pylori treatment previously, and none had received treatment for their ulcer in the week **before** trial entry. 28 patients (13 patients taking OBMT and 15 patients taking BTM) were excluded **after** randomisation (table 2) and 132 patients completed the trial.

Endoscopy done 4 weeks **after** cessation of treatment showed that duodenal-ulcer healing was almost the same in the two groups; 63 (94%; 95% CI 88-100%) of 67 patients who received OBMT had healed ulcers as did 60 (92%; 86-100%) of 65 patients who received BTM. Eradication of H pylori was achieved in 66 (98%; 96-101%) patients who received OBMT compared with 61 (94%; 88-100%) who received BTM (table 3). **During** the first week of treatment, symptoms were relieved more effectively in patients taking OBMT than in those taking BTM. Patients who took OBMT had a mean of 2.9 days (2.3-3.5) **during** which they had pain compared with 4.2 days (3.6-4.8) for patients who received BTM. Subsequently, the number of days **during** which patients had pain was similar (table 4). 48 of the 65 who received BTM took antacids **during** weeks 2-5; the median number of tablets taken was 17 per patient. On an intention-to-treat basis (excluding those who were H pylori negative at the outset), ulcer healing occurred in 91.7% (85.3-98.1%) of OBMT patients and 92.8% (86.6-98.9%) of BTM patients. Eradication of H pylori was achieved in 98.6% (95.9-100%) of patients who received OBMT and 91.3% (84.7-98.0%) of patients who received BTM. **During** the first week of

treatment, patients who took OBTM had 2.8 days (2.3-3.4) of pain compared with 4.3 days (3.7-4.8) for those who took BTM.

The results of testing for H pylori **after** treatment showed complete agreement between smear microscopy, culture, and presence of urease in all but 2 patients (1 false-negative urease, 1 false-negative smear). All isolates were sensitive to tetracycline. 17 were sensitive to metronidazole; a further 52 had reduced sensitivity and 63 were resistant. Isolates obtained from all 5 patients in whom H pylori was not eradicated showed no difference in metronidazole sensitivity from pretreatment isolates; isolates were resistant to metronidazole in 4.

Discussion

Our trial provides evidence that eradication of H pylori allows duodenal ulcers to heal without the need for additional ulcer-healing treatment. The trial was not conducted in a double-blind manner. Because the major end points (ulcer healing and H pylori eradication) are objective, we did not feel it necessary to blind patients to the medication. Follow-up endoscopy and the laboratory tests were, however, done by personnel unaware of the treatment. Our trial supports the hypothesis that H pylori and duodenal ulcer are linked causally rather than by association and confirms that H pylori is an important factor in the pathogenesis of duodenal ulcer.

Randomised trials have already shown that eradicating H pylori reduces ulcer recurrence.[2] In these trials, ulcer healing (achieved by [H.sub.2] blockers or 4-6 weeks of bismuth) occurred independently of whether H pylori was cleared. Nonetheless, several studies have suggested that eradication H pylori not only prevents ulcer recurrence but also aids ulcer healing. Zheng et al[8] randomised 70 patients with peptic ulcer (57 duodenal) to receive furazolidine alone for 2 weeks or placebo. Ulcer healing (all types) occurred in 73% and 24% of patients, respectively, **after** 2 weeks of treatment. H pylori status was not recorded. Graham et al[5] randomised 105 patients with duodenal ulcer to receive ranitidine plus bismuth, tetracycline, and metronidazole, or ranitidine alone. **After** 4 weeks, 74% and 53% of patients, respectively, had healed ulcers; **after** 16 weeks these figures rose to 98% and 84%, respectively. Since H pylori was eradicated in most patients who received triple therapy, the authors concluded that anti-H pylori treatment may be particularly useful for the patient with resistant ulcer. Wagner et al[6] studied 59 patients with ulcers which remained unhealed **after** 6 weeks' treatment with an [H.sub.2] blocker. Patients received either bismuth or ranitidine, or both, until their ulcer healed. **After** 8 weeks, greatest ulcer healing occurred in patients who had taken bismuth. In this group, eradication of H pylori was associated with 86% of ulcers healing compared with 65% if H pylori persisted. Since 8 weeks' treatment with bismuth can heal ulcers without eradicating H pylori, it is difficult to assess how important the eradication of H pylori was in these patients with resistant ulcers.

Our choice of drugs was based on a previous study[7] in which H pylori was eradicated in 94% of patients given this 1-week anti-H pylori regimen. It could be argued that since bismuth heals ulcers independently of its anti-H pylori action, its inclusion confuses our results. This seems unlikely since we gave bismuth for only 1 week; previous studies show that ulcer healing following 2 weeks of bismuth treatment is 35%, [9] **after** 4 weeks is 84-90%, and only reaches 97-100% **after** 8 weeks. [10,11] No antacids were given in the first week to any patient because both omeprazole[12] and bismuth[10] reduced ulcer pain. We also wished to avoid possible antacid interactions with tetracycline. Our subsequent use of antacids in BTM patients only was based on evidence that omeprazole abolishes ulcer pain within 7 days in the majority of patients and therefore antacids would not be needed in patients receiving omeprazole for 4 weeks. Our results showed that omeprazole plus BTM abolished ulcer pain more rapidly than BTM **during** the first week. In subsequent weeks the number of days **during** which pain was experienced was similar in both groups of patients.

Side effects from our regimen resulted in 10 patients failing to take all the treatment and a further 8 reported mild side effects but finished treatment. Previous attempts to find a more simple and effective regimen that eliminates H pylori have invariably resulted in lower eradication rates. Omeprazole with amoxicillin has been investigated but the results are variable. Eradication appears to be proportional to the dose of omeprazole used, being about 30% when 20 mg per day is taken, [13] 50% with

40 mg per day, [14] and rising to 80 mg per day. [15] In the last study, side effects were infrequent but the duration of treatment was 7 1/2 weeks. Previous studies have shown the minimum inhibitory concentration (MIC) for amoxicillin against *H pylori* to be directly related to pH; a low pH necessitates a higher MIC. [16] The same studies showed that the activities of metronidazole and tetracycline against *H pylori* were less dependent on pH. This may partly explain why our regimen (which contained tetracycline rather than amoxicillin) was effective in eradicating *H pylori* regardless of whether acid had been suppressed by omeprazole.

Patients with bleeding ulcers were excluded from this study. We have already observed that only 73% of patients with bleeding ulcers have *H pylori* compared with 95-100% of patients with non-bleeding ulcers. [7] We believe our BTM regimen would be safe in patients who had bled provided that *H pylori* was confirmed, since neither omeprazole nor [H.sub.2] blockers have been shown to affect early rebleeding rates [17] and their omission would therefore make little difference to the final outcome.

Routine eradication of *H pylori* in patients with duodenal ulcer to prevent recurrence has been viewed with reservation for a number of reasons. These include lack of chronicity in some patients, side effects of treatment, ulcers due to nonsteroidal anti-inflammatory drugs, the proven safety of long-term antisecretory agents, and the appearance of bacteria resistant to antibiotics. Resistance to metronidazole has been argued as a reason for failure of pretreatment. Logan et al reported metronidazole resistance in 70% of pretreatment isolates and 100% of post-treatment isolates from patients in whom *H pylori*-eradication treatment had failed. [18] However, 19% of resistant isolates were eradicated by their metronidazole-containing regimen. In another large study, 27% of patients with isolates resistant to metronidazole were cleared of *H pylori* by a regimen of triple therapy. [19] We agree that treatment failure is more likely to be associated with metronidazole resistance, but would stress the poor negative predictive value of such resistance with respect to eradication. Factors that determine success of treatment other than in-vitro antibiotic sensitivity have yet to be identified.

We conclude that duodenal ulcers associated with *H pylori* will heal without acid suppression if *H pylori* is eradicated. This approach is recommended for patients in whom both ulcer healing and eradication of *H pylori* are required. Combining omeprazole with triple therapy reduces ulcer pain more rapidly, but since it has no additional effect on ulcer healing, administration for more than 1 week appears to be unnecessary.

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Diagnostic value of decreasing IgG , IgA , and IgM antibody titres after eradication of *Helicobacter pylori*.

Kosunen, Timo U.; Seppala, Kari; Sarna, Seppo; Sipponen, Pentti
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ABSTRACT: An assay that measures the levels of the **antibody IgG** in the blood may be an effective method of determining whether patients treated for *Helicobacter pylori* infections are infection-free. *Helicobacter pylori* is a bacteria that infects the digestive tract. Out of 144 patients treated with drugs for *Helicobacter pylori* infections, 121 (84%) were bacteria-free six weeks **after** treatment. At that time, **IgG** levels had decreased 20% to 30% in both the infected patients and the bacteria-free patients. At six and 12 months **after** treatment, **IgG** levels were 50% or less of pretreatment values in 97% of the bacteria-free patients. **IgG** levels remained the same or increased slightly in the 23 infected patients. Levels of the **antibodies IgA** and **IgM** decreased in the bacteria-free patients, and remained the same in the infected patients. **IgG** was a more sensitive indicator of an individual's disease status than **IgA** and **IgM**.

TEXT:

Reports that *Helicobacter pylori* infection is a risk factor for gastric carcinoma [1,2] have increased interest in the use of non-invasive tests to verify eradication of *H pylori* by antibiotic therapy. The two tests in use--urea breath tests [3,4] and serological tests for circulating *H pylori* **antibodies** [5] --are sensitive and specific indicators of the presence of *H pylori* in the stomach. A serological test alone has been reported to be sufficient to detect *H pylori* infection and therefore probably able to replace endoscopy as a primary diagnostic procedure in patients aged under 45 years of age. [6] The scarce follow-up data available indicate that **antibody** titres remain stable **during** infection, [7,8] but that they decline with successful treatment. [8-12] Here we report what drop in **antibody** titres reliably indicates eradication of *H*

pylori and how long it takes for this fall to occur.

SUBJECT AND METHODS

Patients and treatment schedule

144 patients (81 men and 63 women), mean 51-7 years (range 24.9-80), who were examined for gastric disease and shown by histology, bacterial culture, or both to be infected with *H pylori*, were given triple antimicrobial treatment (see below) for 2 weeks, 78 patients had duodenal ulcer, 16 gastric ulcer, 2 duodenal and gastric ulcer, and 48 chronic gastritis. 5 other patients were treated but were excluded from the study--2 dropped out **before** the 6-week samples were taken (1 of them was operated on a year later in another hospital for gastric ulcer and the postoperative histological specimens showed no bacteria); and 3 patients were lost **before** 6-month samples were obtained (1 died and 1 acquired a mental disorder).

The pretreatment and follow-up samples, which were taken 6([+ or -] 2 weeks, 6([+ or =] months, and 12([+ or Months **after** the start of therapy, included biopsy samples for histology and bacterial cultures, and serum samples for circulating *H pylori* **antibodies**. Some patients missed one of the three follow-up appointments. The study protocol was accepted by the ethical committee of the clinic and all patients gave informed consent.

The 2-week treatment consisted of colloidal bismuth subcitrate (120 mg four times daily), metronidazole (500 mg three times daily), and amoxycillin (500 mg 4 times daily). 3 patients were given tetracycline (250 mg four times a day) instead of amoxycillin because of allergy to penicillin.

Investigations

Two mucosal samples were taken from the duodenal bulb, the antrum, and the body for histology, and one from the antrum and corpus for bacterial culture. Haematoxylin-eosin and giemsa stains were used for histological examination. Details of a morphometric analysis of cellular changes in 23 consecutive patients in this group have been reported elsewhere, [13] as will the histological and clinical data of all 144 patients (unpublished). Samples of *H pylori* cultures were taken in 0.5 ml of 20% glucose, ground in mortars, and inoculated into selective and non-selective agar plates within 4 h. [14]

Antibodies to *H pylori* were measured separately for **IgG**, **IgA**, and **IgM** by an enzyme **immunoassay** method. [15] The antigens used were an acid glycine extract [15] and a bacterial sonicate [16] obtained by ultracentrifugation from *H pylori* strain NCTC 11637. The absorbance readings were converted to reciprocals of the end-point titres. The end-point titres were the dilutions of the serum at the cut-off level defined by the optical densities of positive reference serum pools at constant dilutions. Separate reference pools were used for immunoglobulins A, G, and M. They were placed on each microtitre plate and all samples from a patient were studied, in threefold dilutions when needed, on the same plate. The lower limits of raised titres (expressed as reciprocals) were 700 for **IgG**, 100 for **IgA**, and 150 for **IgM antibodies**. The data reported were obtained by use of the acid glycine extract antigen with one exception, when only the sonicate preparation gave raised readings.

Results

Before triple therapy all 144 patients harboured bacteria and had histologically verified active chronic gastritis. All but 1 (99%) had raised titres of circulating *H pylori* **antibodies**. **IgG antibodies** detectable with acid glycine extract were found in 139 (97%) patients. 3 patients had raised titres of only **IgA** against the same antigen and 1 had only **IgG antibodies** detectable with the bacterial sonicate preparation (sensitivity of the **IgG** tests 97%). Raised titres of **IgA antibodies** were found in 92 (64%) and raised titres of **IgM antibodies** in 8 (6%) patients.

Some patients reported minor side-effects from antimicrobial therapy and 4 took only a week's course of therapy (all were treatment failures). Of the few patients in whom self-limiting diarrhea developed, 2 had *Clostridium difficile* cultured from stool samples.

The criteria for eradication of bacteria were absence of bacteria in follow-up biopsy specimens as verified histologically, by culture, or by both means. Bacteria were eradicated from 121 of the 144 patients (84%), but 1 patient

IgG antibody had fallen slightly by 6 weeks in 90% of the bacteria-negative and 60% of the bacteria-positive patients. Falling **IgA**

titres were as common; in only 2 of the 80 bacteria-negative patients did the titre remain at the original value. 6 of the 8 raised IgM titres decreased; 7 of these patients had become bacteria-negative.

In bacteria-negative patients **antibody** titres continued to fall beyond the first 6 weeks, whereas in bacteria-positive patients titres remained high throughout the observation period. The accompanying figure shows the changes (given as medians) in **IgG** and **IgA** titres in patients grouped according to success of eradication of *H pylori*.

6 months **after** therapy **IgG** titres in bacteria-negative patients were average 24% of the pretreatment value and **IgA** titres 34%. Only in 3 patients was the **IgG** titre at 6 months greater than 50% of the pretreatment one. In 1 of these patients the **IgA** titre at 6 months was 33% of pretreatment value, as was the **IgG** titre at 12 months. In the other 2 **IgG** titres remained high (62 and 122%), even at 12 months. In all 3 patients who initially had only raised **IgA** titres, the fall by 6 months was greater than 50% of the pretreatment value. 11 of the 66 samples from initially **IgA** -positive patients showed less than a 50% decrease in **IgA** titre. 4 of the 8 originally IgM-positive patients had a less than 50% drop in this titre at 6 months; 1 of them was still bacteria-positive. For bacteria-positive patients, titres at 6 months were 57-109% of pre-treatment values for **IgG** and 47-200% for **IgA**.

12-month samples from bacteria-negative patients showed that **antibody** titres in all immunoglobulin classes declined continuously. In all but 3 patients the fall in **IgG** titre was 50% or greater; in these 3 the **IgA** titre was initially low. 3 of the 59 originally **IgA** -positive patients from whom

[TABULAR DATA OMITTED]

12-month samples were available had a less than 50% drop in this titre (for IgM it was 1 out of 6). All 3 patients were among those whose **IgG** titre at 12 months was less than 50% of initial titre. 4 patients who were culture negative at 6 weeks and 6 months were not willing to undergo further endoscopy. Their **antibody** titres continued to decline and were well within the range of those who underwent endoscopy. Most patients who were bacteria-positive at 6 months had had a second course of triple therapy, so there were only 11 bacteria-positive patients at 12 months; their **IgG** titres at 12 months were 75-129% of the initial value. 7 of them were initially **IgA** positive and all have remained so (range 73-150%). A patient who was reinfected **before** the 12-month visit had had, at 6 months, an **IgG** titre of 20%, and an **IgG** titre of 32%, less than the original titre, but at 12 months titres of both **antibodies** approached the initial level.

The predictive values of decreased **IgG** and **IgA** titres at different times **after** treatment are in the accompanying table. The sensitivity of a 50% fall in **IgG** titre as an indicator of bacterial eradication is 12% at 6 weeks. At 6 months, the sensitivity is already 97%, and the decrease is 95% specific. At 6 months, the sensitivity of a 50% decrease in **IgA** titres is 84% and the specificity 90%. From the 6th month on the predictive value of a fall in **antibody** titre to less than 60% of pretreatment value is at least 0.98 for both **IgG** and **IgA**. Titres of 40% or less of the pretreatment value were seen only in bacteria-negative patients. of **IgG antibodies**. [8-12] Our data confirm these findings and further show, in a group of 121 adult patients, that (1) the falls in **IgG** titres persist **after** eradication of bacteria; (2) the same is true for **IgA** and IgM **antibodies**, whether or not there is a concurrent **IgG** response; and (3) when end-point titres are used for quantifying **antibodies**, a 60% or greater decrease in either **IgG** or **IgA** titres confirms eradication of *H pylori*. Even a 20% decrease in titres is a very dependable indicator of healing since only 2 to 23 patients remaining infected had this degree of drop 6 months **after** therapy. Ariel 3 successfully treated, initially **antibody** -positive patients did not have this big drop in **IgG** titre. Further microbiological and histological studies of these patients are needed to determine whether the bacteria have been eradicated fully or only to undetectable levels.

In most patients who were treated with antimicrobial drugs, there was an initial fall in **antibody** titres irrespective of the final bacteriological result. In patients who were therapeutic failures, the **antibody** titres then remained stable or rose. In the patient who was reinfected **after** the 6-month visit, **IgG** and **IgA** titres that had fallen sharply showed a sharp rise at 12 months.

Our results indicate that changes in **IgG** and **IgA** titres offer an easy means of monitoring the disappearance and reappearance of *H pylori* in the human stomach. Changes in **IgA** titres were not as consistent as those of **IgG** titres; initially **IgA** -positive patients did not show a 50% decrease in this titre despite eradication of *H pylori* 6 months earlier. However, all but 1 of them had a 50% or greater fall in **IgG** titre. The main value of **IgA** titres is in diagnosis and follow-up of patients who do not produce an **IgG** response but who have raised **IgA** titres; in our study group 2% fell into this group. **IgM** responses were always accompanied by changes in **IgG** titres, which limits their value in follow-up studies. They may be more useful in acute infection and exacerbations of chronic infections. [12]

Since a fall in circulating *H pylori* **antibodies** is accompanied by healing of the inflammatory changes in the stomach wall **after** eradication of *Helicobacter*, [13,17] we have a cheap and ethical non-invasive method for use in follow-up studies. Breath tests [3,4] are another means of verifying the success of therapy and may be applied a few weeks earlier than serological tests, but they require special equipment, time personnel to collect the breath samples, and the use of either radioactive material or tracers that can be measured only with mass spectrometers.

The use of serological tests will be useful in the assessment of healing in individual patients; in large epidemiological studies aimed at finding out the influence of cure of active chronic gastritis on other diseases associated with it, and on the development of atrophic gastritis, for example; [18] in the follow-up of patients with duodenal ulcers; and in monitoring efficacy of triple therapy.

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SPECIAL FEATURES: illustration; table; graph

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One week eradication regimen for Helicobacter pylori.

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ABSTRACT: The bacteria Helicobacter pylori is a cause of stomach inflammation and a risk factor for the recurrence of duodenal ulcers. Although these conditions are very common, no treatment program has yet been found which both safely and effectively eradicates infection with this bacteria. However, researchers have now developed a treatment regimen which is rapidly successful in eliminating in most patients with H. pylori infection. A total of 106 people infected with H. pylori participated in the study; this included both patients with peptic ulcers and those with upset stomachs but without ulceration. The treatment regimen consisted of three drugs. Tripotassium dicitrato bismuthate, which coats the stomach lining, was given at 120 milligrams, four times a day. Amoxicillin, an antibiotic, was administered at 500 milligrams, four times a day. Both of these compounds were given for seven days; **during** the last three days, metronidazole (another antibiotic) was given at 400 milligrams, five times daily. The infection with Helicobacter pylori was successfully eliminated in 76 of the 106 patients. The percentage of elimination was better among the 43 patients who were found to be infected with a metronidazole-sensitive strain of bacteria. Among these patients, 40 (93 percent) were cured of their infection. The most common side effect of treatment was a taste disturbance, resulting from the metronidazole, which affected 24 patients. This study shows that it is possible to eliminate H. pylori infection in many cases with a one-week regimen that is safe and inexpensive. (Consumer Summary produced by Reliance Medical Information, Inc.)

TEXT:

Introduction

Helicobacter pylori is a common cause of chronic gastritis [1] and an important factor in duodenal ulcer recurrence. [2] Eradication of infection might be indicated in patients with duodenal ulcer disease. [3] Bismuth salts, given to patients with dyspepsia and peptic ulcer, [4] are bactericidal against H pylori, but do not penetrate into the gastric pits

that might act as sanctuary or reservoir sites for *H pylori*. Thus, even **after** a one month course of bismuth, *H pylori* is eradicated in only 10% of patients and recurs in most subjects within days. [5] Triple therapy, bismuth combined with metronidazole and another antibiotic, is more effective but not ideal because of the risks of side-effects, drug resistance, and poor compliance.

We have previously shown that treatment with bismuth for one week is as effective as for four weeks; [6] longer treatment regimens should not be needed. This study assesses the efficacy of one-week triple therapy in eradicating *H pylori* infection.

Patients and methods

After routine diagnostic upper gastrointestinal endoscopy, patients with *H pylori* infection were invited to enter the study, which was approved by the Parkside ethical committee. Patients gave written consent. Those subjects with previous gastric surgery, known bleeding diathesis, on oral anticoagulants, or who had taken bismuth compounds or antibiotics known to be active against *H pylori* within the previous two months, were excluded.

All endoscopes were disinfected **after** each examination with an automatic washing machine (Olympus EW20) [7] and the biopsy forceps were sterilised by autoclaving.

H pylori status

The presence of *H pylori* was assessed by the [sup.13.C-urea] breath test ([sup.13.C-UBT]), histological examination of two antral biopsy specimens, and culture (two antral specimens, selective and non-selective media, and microaerophilic conditions for up to 10 days). Where possible, pure cultures were harvested and stored in 10% glycerol broth at -- 80[degrees]C. Tests for metronidazole sensitivity were completed by an in-vitro disk method (Mast sensitivity disks, Mast Laboratories, Liverpool, UK).

Patients were classified as *H pylori* positive by a positive [sup.13.C-UBT] together with positive histology or culture, and *H pylori* negative by a negative [sup.13.C-UBT], negative histology, and negative culture to confirm eradication. Recurrence **after** eradication was monitored by the [sup.13.C-UBT].

Clearance of *H pylori* was defined as a negative [sup.13.C-UBT] at the end of treatment. Eradication was defined as a negative [sup.13.C-UBT], negative histology, and negative culture one month **after** the end of treatment.

[sup.13.C-urea] breath test

The [sup.13.C-UBT] (European Standard Protocol) [8] was completed within two days of the initial endoscopy in all patients. A baseline sample of expired breath was obtained **before** drinking a fatty liquid test meal (76% lipid, 19% carbohydrate, 5% protein) to delay gastric emptying. **After** 10 min, 100 mg [sup.13.C-urea] (99% pure, Cambridge Isotopes, Boston, USA) in 50 ml tap water was swallowed and distributed within the stomach by turning the patient to the left and right decubitus position. 2 litre serial breath samples were collected every 5 min into a large reservoir collecting bag, from which a single 20 ml sample was taken at the end of the test and analysed by mass spectrometry (BSIA, Brentford, London). A positive result was defined as excess, [sup.13.CO.sub.2] excretion greater than 5 per mil. [8,9]

Treatment

Patients with a positive [sup.13.C-UBT] and positive histology or culture were given tripotassium dicitrate bismuthate 120 mg four times daily and amoxycillin 500 mg four times daily for seven days (days 1-7). Erythromycin 500 mg four times daily was given to 2 patients who were allergic to penicillin. To decrease the risk of promoting metronidazole-resistant *H pylori*, metronidazole 400 mg five times per day was given for the last three days (days 5-7).

A second [sup.13.C-UBT] was completed immediately **after** the end of treatment when compliance and side-effects were assessed by returned tablet count and direct questioning, respectively. A third [sup.13.C-UBT] and second endoscopy with biopsies were done one month later, with negative culture, histology, and [sup.13.C-UBT] taken to indicate successful eradication.

Follow-up clinical assessments and [sup.13.C-UBT] were done at 3, 6, and 12 months **after** the end of treatment to monitor recurrence of either *H pylori* or symptoms.

Results

106 patients (60 male, median age 45 years, range 16-80) entered the study. Indications and/or findings at endoscopy included a previous history of duodenal ulcer (n=60), non-ulcer dyspepsia (n=42), or gastric ulcer (n=4). Every patient had H pylori detected by histological examination of antral biopsy specimens and a positive [¹³C-UBT] (mean [range] pretreatment excess [Δ]¹³CO.sub.2 excretion, 28.0 [6.2-87.4] per ml). H pylori was successfully cultured in 84/106 (79%) patients. Immediately **after** finishing treatment, the [¹³C-UBT] was negative in all patients (mean [range] excess [Δ]¹³CO.sub.2 excretion, 1.9 [0.2-4.9] per ml). H pylori was successfully eradicated in 76/106 (72%) patients one month **after** the end of treatment (mean [range] excess [Δ]¹³CO.sub.2 excretion, 1.7 [0.2-3.4] per ml), which was confirmed by negative antral histological examination and culture. Patients who had H pylori successfully eradicated one month **after** completing treatment remained negative thereafter, with no evidence of H pylori recurrence **during** subsequent follow-up with [¹³C-UBT] (fig 1).

In the remaining 30 (28%) patients, the [¹³C-UBT] became positive with symptomless recurrence at one month in 28 subjects (mean [range] excess [¹³CO.sub.2 excretion, 15.6 [6.4-34.0] per ml). In 2 patients with duodenal ulcer, dyspeptic symptoms recurred **before** the one month follow-up: both had positive [¹³C-UBT], histology, and culture for H pylori, with recurrence of duodenal ulcer confirmed at endoscopy two weeks **after** the end of treatment.

In those patients known to have a metronidazole-sensitive strain of H pylori (n=43), the eradication rate was 93% (40/43) (fig 2).

Compliance and side-effects

Every patient completed the one-week treatment, although 3 patients mistakenly took metronidazole on days 1-3 instead of 5-7. The commonest side-effects were taste disturbance (24%) and loose stools (20%). No patient had severe diarrhoea. Oral candidosis (n=1) and vaginal moniliasis (n=1) were mild and resolved spontaneously. In 3 patients with pre-treatment metronidazole-sensitive strains of H pylori, the microorganism was not eradicated despite all 3 apparently complying with their medication.

Metronidazole sensitivity

In every patient in whom eradication failed (n=30), positive cultures of H pylori from antral biopsy specimens obtained one month **after** finishing treatment were resistant to metronidazole. Of 21/30 pretreatment cultures taken from the same patients (successfully regrown from storage at -- 80[degrees]C), 17/20 (85%) were found to be resistant to metronidazole.

By contrast, of 44 pretreatment cultures successfully regrown from storage from the 76 patients in whom H pylori was eradicated, the organism was resistant to metronidazole in only 4 (p < 0.001; [χ^2]). Attempts to regrow H pylori from storage failed in 20 patients. Only 3 patients with metronidazole-resistant H pylori had previously taken metronidazole. Resistant strains were equally common for both men and women and in Europeans and non-Europeans (fig 3).

Discussion

Eradication of H pylori in patients with duodenal ulcer is likely to decrease the subsequent rate of recurrence. [2,3] Successful eradication has usually been achieved by lengthy triple therapy with antibiotics and bismuth, which may be poorly tolerated and cause serious side-effects. [10,11] Patients' compliance, [12] often impaired because of side-effects and length of treatment, is an important factor in determining the success rate of any drug regimen. In our study, all patients were informed in advance that both taste disturbance while taking metronidazole and increased stool frequency may occur; this may have contributed to the good compliance that we observed.

Our one-week regimen led to a 72% eradication rate, which compares favourably with the older and longer regimens advocated at present. All patients had negative [¹³C-UBT] at the end of treatment, indicating that the organism had been suppressed. Recurrence of metronidazole-resistant H pylori, presumably from sanctuary sites, occurred within one month in 30 patients, whereas H pylori was successfully eradicated in the remainder.

H pylori has not recurred in any of the patients in whom it was eradicated (mean follow-up 9.4 months). In previous studies, premature assessments of H pylori status **after** finishing treatment--eg, by adopting only biopsy-based methods--may have missed small quantities of recurrent H

pylori, so that further apparent recurrences were recorded only **during** subsequent follow-up at three and six months. [13] Such recolonisation was not missed with the semi-quantitative [sup.13.C-UBT], and all recurrent H pylori were detected within one month **after** the end of treatment.

The efficacy of any regimen that is designed to eradicate H pylori depends on both metronidazole sensitivity [14] and compliance with treatment, [12] factors that have not been prospectively studied in previous reports. The outcome of this study suggests that pretreatment metronidazole resistance is a major influence on the eradication rate of any therapeutic regimen that includes metronidazole. Analysis of results in the subgroup of patients with metronidazole-sensitive H pylori shows that the eradication rate with one week of treatment was 93%. Others have reported eradication rates of up to 79% with bismuth and metronidazole alone, but since metronidazole sensitivity was not determined **after** treatment, the treatment itself may have promoted the emergence of metronidazole-resistant H pylori. [15] For this reason, triple therapy with amoxycillin was given. The dose of metronidazole in our study was 400 mg five times per day, higher than that normally used, but this was sufficient to overcome resistance to metronidazole in only 4/21 (19%) of patients. Higher doses of metronidazole, even with a second antibiotic, do not seem to be effective against resistant organisms. The effects of different durations of treatment with metronidazole were not studied.

The frequency of metronidazole-resistant strains of H pylori might therefore largely determine the eradication rate of any therapy that does not initially exclude patients with metronidazole-resistant H pylori. This observation has wide-reaching and important implications. Treatment of patients in areas where metronidazole-resistant H pylori is common should ideally await metronidazole-sensitivity testing **before** triple therapy is prescribed. Although this practice would increase the complexity and cost of treatment, it would prevent unnecessary exposure to antibiotics that will not eradicate H pylori. In Central Africa, up to 94% of the population have metronidazole-resistant H pylori, [16,17] and our study suggests a poor outcome for attempts to control both duodenal ulcer relapse and gastritis (which may influence the frequency of intestinal-type gastric cancer [18,19]) by antimicrobial agents for H pylori. In the UK, 20% of strains of H pylori are resistant to metronidazole probably because of previous use. [14]

Our results indicate that antral biopsies should routinely be taken **before** treatment for in-vitro metronidazole-sensitivity testing in patients in whom H pylori eradication therapy is indicated. These data suggest that in patients colonised by metronidazole-sensitive H pylori, the shortened therapeutic regimen tested here will be highly effective, with an eradication rate of 93%.

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CAPTIONS: 13C-urea breath test **before** and **after** treatment. (graph); Pretreatment metronidazole-sensitivity tests. (chart)

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SPECIAL FEATURES: illustration; graph; chart

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Symptomatic Benefit from Eradicating *Helicobacter pylori* Infection in Patients with Nonulcer Dyspepsia (Original Articles)

McColl, Kenneth; Murray, Lilian; El-Omar, Emad; Dickson, Anthea; El-Nujumi, Adil; Wirz, Angela; Kelman, Andrew; Penny, Christine; Knill-Jones, Robin; Hilditch, Thomas.

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CORPORATE SOURCE: From the Departments of Medicine and Therapeutics (K.M., L.M., E.E.-O., A.D., A.E.-N., A.W., A.K.) and Clinical Physics (T.H.), Western Infirmary; Victoria Infirmary (C.P.); and the Department of Public Health, University of Glasgow (R.K.-J.) -- all in Glasgow, United Kingdom. Address reprint requests to Dr. McColl at the Department of Medicine and Therapeutics, Western Infirmary, Glasgow G11 6NT, United Kingdom.

Abstract

Background: The eradication of *Helicobacter pylori* infection is beneficial in patients with gastric or duodenal ulcers. The value of eradicating the infection in patients with dyspepsia and no evidence of ulcer disease is not known.

Methods: We performed a randomized, placebo-controlled trial comparing the efficacy of treatment for two weeks with 20 mg of omeprazole orally twice daily, 500 mg of amoxicillin three times daily (with 500 mg of tetracycline three times daily substituted for amoxicillin in patients allergic to penicillin), and 400 mg of metronidazole three times daily (160 patients) with that of omeprazole alone (158 patients) for resolving symptoms of dyspepsia in patients with *H. pylori* infection but no evidence of ulcer disease on upper gastrointestinal endoscopy. Symptoms were assessed with the Glasgow Dyspepsia Severity Score, with resolution of symptoms defined as a score of 0 or 1 in the preceding six months (maximal score, 20). One year later the patients were assessed to determine the frequency of the resolution of symptoms.

Results: One month **after** the completion of treatment, 132 of 150 patients (88 percent) in the group assigned to receive omeprazole and antibiotics had a negative test for *H. pylori*, as compared with 7 of 152 (5 percent) in the group assigned to receive omeprazole alone. One year later, dyspepsia had resolved in 33 of 154 patients (21 percent) in the group given omeprazole and antibiotics, as compared with 11 of 154 (7 percent) in the group given omeprazole alone (95 percent confidence interval for the difference, 7 to 22 percent; $P < 0.001$). Among the patients in the group given omeprazole and antibiotics, the symptoms resolved in 26 of the 98 patients (27 percent) who had had symptoms for five years or less, as compared with 7 of the 56 patients (12 percent) who had had symptoms for more than five years ($P = 0.03$).

Conclusions: In patients with *H. pylori* infection and nonulcer, or functional, dyspepsia, treatment with omeprazole and antibiotics to eradicate the infection is more likely to resolve symptoms than treatment with omeprazole alone. (N Engl J Med 1998;339:1869-74.)

TEXT

Dyspepsia affects 20 to 40 percent of the population of the **Western** world. (Ref. 1-4) Upper gastrointestinal endoscopy of patients with dyspepsia reveals no abnormality in the majority; such patients are considered to have nonulcer, or functional, dyspepsia. (Ref. 5) The cause of nonulcer dyspepsia is unclear but is thought to be heterogeneous. (Ref. 6-8) The management of the disorder is unsatisfactory, and there are few studies showing that active treatment is superior to a placebo. (Ref. 7)

The recognition of the pathogenic role of *Helicobacter pylori* infection in patients with peptic ulcer and the benefits of eradicating the infection (Ref. 9-11) has led to suggestions that the infection may also be the cause of dyspepsia in some patients with nonulcer dyspepsia. A higher prevalence of *H. pylori* infection has been reported in patients with dyspepsia than in those without it, but in some of these studies the patients were not matched for age or excluded if they had underlying ulcer disease. (Ref. 12-14) Several studies have examined the effect of eradicating *H. pylori* infection on dyspeptic symptoms in patients with nonulcer dyspepsia, but the results have been conflicting and all such studies have been criticized because of design flaws. (Ref. 15) In a recent review of these trials, Talley and Hunt concluded that "*H. pylori* has not been established, as yet, to play a definitive role in dyspepsia." (Ref. 15)

We report the results of a large, randomized, placebo-controlled trial that assessed the effect of eradicating *H. pylori* in patients with nonulcer dyspepsia.

Methods

Recruitment and Evaluation of Patients

We studied patients who were referred to one dyspepsia clinic by their primary care physician because they had had dyspepsia for at least four months. All patients were found to have *H. pylori* infection but no endoscopic evidence of current or previous peptic ulcer disease. Dyspepsia was defined as intermittent or persistent pain or discomfort in the upper abdomen or lower part of the chest, heartburn, nausea, a feeling of postprandial fullness, or any other symptoms thought to be related to the upper gastrointestinal tract. (Ref. 16) Patients were excluded if they had previously been found to have peptic ulcer disease, had endoscopic evidence of esophagitis, were taking nonsteroidal antiinflammatory drugs (other than low-dose aspirin), had undergone gastric resection, were pregnant, or had previously been treated for *H. pylori* infection.

The patients had been asked to stop taking any antisecretory drug

before the initial clinic visit. At this visit, a standardized interview was used to determine each patient's symptoms and the duration of symptoms (less than 2 years, 2 to 5 years, more than 5 to 10 years, or more than 10 years) and a physical examination was conducted.

The severity of the dyspepsia **during** the six months preceding the visit was assessed with the Glasgow Dyspepsia Severity Score. (Ref. 17) This scale evaluates the frequency of symptoms (maximal score, 5); the effect of dyspepsia on normal activities (2); the number of days of work missed because of dyspepsia (2); and the frequency of medical consultations (2), home visits by a physician (2), tests for dyspepsia (2), use of over-the-counter medications (2), and use of prescribed medications (3). Scores can range from 0 to 20, with higher scores indicating more severe dyspepsia. The quality of life was assessed with the 36-item Medical Outcomes Study Short-Form General Health Survey (SF-36), which examines eight aspects of the quality of life: general and mental health, physical function, social function, physical and emotional health, pain, and vitality. Scores on each of the eight aspects can range from 0 (worst) to 100 (best). (Ref. 18)

After the clinical assessment, the patients underwent a carbon-14 urea breath test to determine their *H. pylori* status. (Ref. 19) The results of the test were considered positive if the value at 20 minutes was more than 30 (units equal the percentage of the dose administered per millimole of carbon dioxide expired times the body weight in kilograms times 100). A blood sample was also taken to determine whether IgG antibodies to the CAGA gene product of *H. pylori* were present (test kindly performed by Dr. Jean Crabtree). The presence of these antibodies indicates that patients are infected with a more virulent strain of the bacterium. (Ref. 20)

Two weeks later the patients underwent upper gastrointestinal endoscopy. **Before** endoscopy, those with a positive urea breath test were invited to undergo randomization to either treatment to eradicate *H. pylori* or placebo treatment if the endoscopy showed no evidence of ulcer disease or esophagitis and to undergo studies of gastric acid secretion **after** the endoscopic examination. The randomization was carried out independently by the pharmacy department, which used tables of random numbers to assign half the patients to receive active treatment with omeprazole, amoxicillin, and metronidazole and half to receive omeprazole and placebo. The active and placebo tablets for each antibiotic were identical in appearance, and the trial was conducted in a double-blind fashion. The study was approved by the Western Infirmary ethics committee, and all patients gave written informed consent.

During the upper gastrointestinal endoscopy, biopsy samples were obtained from the antrum and body of the stomach. The samples were sectioned and stained with hematoxylin and eosin and examined for *H. pylori*. They were also examined for bacterial urease with a urease slide test (CLO test, Delta West, Bentley, Australia). The patients who had agreed to undergo acid secretory studies had a nasogastric tube inserted immediately **after** the removal of the endoscope, and they were then taken to the recovery area and allowed to rest for one hour. Acid secretion was then measured first in response to a one-hour intravenous infusion of gastrin-releasing peptide (Calbiochem Novabiochem, Nottingham, United Kingdom) at a dose of 40 pmol per kilogram of body weight per hour and then in response to a one-hour intravenous infusion of pentagastrin (Zeneca, Macclesfield, United Kingdom) at a dose of 0.6 microg per kilogram per hour.

After these tests, the patients received a two-week course of either 20 mg of omeprazole (Losec, Astra Hassle, Molndal, Sweden) twice daily, 500 mg of amoxicillin three times daily, and 400 mg of metronidazole three times daily or 20 mg of omeprazole twice daily and placebo antibiotics. Patients with a history of allergy to penicillin were given 500 mg of tetracycline three times daily rather than amoxicillin.

The patients were given an appointment for a urea breath test and an assessment of side effects four weeks **after** treatment was completed and an appointment for a full reassessment one year **after** the start of treatment. They were also told that they could take any medication necessary, including a proton-pump inhibitor, if they had recurrent or persistent symptoms **after** the completion of treatment. However, their doctors were requested by letter not to prescribe any treatment to eradicate *H. pylori*. At the one-year visit, dyspeptic symptoms **during** the preceding six months were assessed with the Glasgow Dyspepsia Severity

Score, the quality of life was assessed with the SF-36 questionnaire, and *H. pylori* status was assessed with a urea breath test. The results of the breath tests performed one month **after** the completion of treatment and one year **after** the start of treatment were not divulged to the patients or investigators until **after** the completion of the one-year assessment.

Statistical Analysis

The main end point was the resolution of symptoms, defined as a score of 0 or 1 on the Glasgow Dyspepsia Severity Score, because validation studies indicated that this cutoff point was the most appropriate indication of the resolution of dyspepsia. (Ref. 17) The analysis was based on the intention-to-treat principle. Two-sample t-tests were used to compare the mean values of variables considered continuous in the active-treatment and placebo groups. Chi-square tests were used to analyze categorical variables. A logistic-regression analysis was carried out to determine which characteristics of the patients were predictive of the resolution of symptoms. All statistical tests were two-sided.

Results

Between October 1994 and October 1996, we assessed 916 patients with dyspepsia, of whom 330 were enrolled in the study. The reasons for exclusion were endoscopic evidence of duodenal or gastric ulcer or esophagitis (209 patients), a negative test for *H. pylori* (306 patients), previous treatment for *H. pylori* (21 patients), inability to tolerate endoscopy (11 patients), use of nonsteroidal antiinflammatory drugs (9 patients), and pregnancy or a serious medical condition (21 patients). Of the eligible patients, only nine declined to participate.

Of the 330 patients who enrolled in the study, 12 were excluded from the final analysis. Eleven did not have at least two positive tests (Ref. 21) for *H. pylori* infection, because they had entered the study immediately **after** the endoscopic examination when the results of the urea breath test were available but the results of the urease test and histologic analysis were not available; these subsequently proved to be negative. One patient did not meet the criteria for nonulcer dyspepsia because his base-line Glasgow Dyspepsia Severity Score in the six months preceding the study was 0.

Of the remaining 318 patients, 160 were assigned to receive therapy to eradicate *H. pylori* (omeprazole and antibiotics) and 158 were assigned to receive omeprazole alone. The two groups were well matched with respect to age, sex, initial results on the Glasgow Dyspepsia Severity Score, prevalence of previously prescribed antisecretory-drug therapy, prevalence of smoking, predominant symptom at presentation, duration of symptoms, and quality of life (Table 1).|*Table 1.-Base-Line Characteristics of 318 Patients with Nonulcer Dyspepsia *.**TABLE OMITTED***

A total of 308 patients (97 percent) were reassessed one year **after** randomization, 154 in each group. Of the 154 patients in the group assigned to receive omeprazole and antibiotics, 150 had a urea breath test one month **after** the completion of treatment. The test was negative in 132 patients (88 percent), of whom 125 (95 percent) also had a negative test at one year. Of the 154 patients in the group assigned to receive omeprazole alone, 152 had a urea breath test one month **after** the end of treatment. The test was positive in 145 patients (95 percent), of whom 134 (92 percent) also had a positive test at one year.

The distribution of Glasgow Dyspepsia Severity Scores in the two groups at one year is shown in Figure 1. On an intention-to-treat basis, the symptoms resolved (defined as a score of 0 or 1) in 33 of 154 patients (21 percent) in the group assigned to receive omeprazole and antibiotics, as compared with 11 of 154 patients (7 percent) in the group assigned to receive omeprazole alone (95 percent confidence interval for the difference between groups, 7 to 22 percent; $P<0.001$).|*Figure 1.-Distribution of Glasgow Dyspepsia Severity Scores One Year **after** the Start of Treatment.The resolution of symptoms (defined as a score of 0 or 1) was significantly more common in the group assigned to receive omeprazole and antibiotics ($P<0.001$). Data were missing for one patient in the omeprazole-alone group *.**FIGURE OMITTED***

The mean (\pm SD) dyspepsia score at one year was 5.4 ± 4.0 in the group treated with omeprazole and antibiotics, as compared with 6.2 ± 3.6 in the group treated with omeprazole alone ($P=0.07$) (Table 2). The scores in both groups were lower than those at base line; however, the higher scores at entry were due in part to the fact that all patients received a total of three points for visits to their general practitioner, visits to

the hospital, and undergoing endoscopy. | *Table 2.-Outcomes of the Patients at One Year *.**TABLE OMITTED***

At base line, 84 percent of the patients in the group given omeprazole and antibiotics and 80 percent of those in the group given omeprazole alone had been taking prescription drugs for dyspepsia **during** the preceding six months (Table 1). At one year, the respective values were 43 percent and 53 percent (Table 2). At base line, among patients who were taking prescription drugs for dyspepsia, 53 percent of the patients in the group given omeprazole and antibiotics had taken such a drug for more than three of the preceding six months, as compared with 55 percent of the patients in the group given omeprazole alone; at one year the respective values were 47 percent and 57 percent. There was no significant difference between groups in the quality of life **after** treatment (Table 2). We used univariate logistic-regression analysis to examine whether any characteristics of the patients at base line could be used to predict a response to treatment. Only the duration of symptoms was predictive ($P=0.03$). None of the following factors were predictive: age ($P=0.81$), sex ($P=0.20$), smoking status ($P=0.80$), initial Glasgow Dyspepsia Severity Score ($P=0.35$), predominant symptom at presentation ($P=0.50$), *H. pylori* CagA status ($P=0.78$), acid secretion in response to pentagastrin ($P=0.92$), or acid secretion in response to gastrin-releasing peptide ($P=0.35$). In a multivariate model, treatment ($P<0.001$) and the duration of symptoms ($P=0.03$) were independently related to the resolution of symptoms. There was no evidence of an interaction between treatment and the duration of symptoms ($P=0.90$). The odds ratio for the resolution of symptoms in the group assigned to receive omeprazole and antibiotics, as compared with the group assigned to receive omeprazole alone, was 3.9 (95 percent confidence interval, 1.8 to 8.3). The percentage of patients with resolution of symptoms decreased with increasing duration of symptoms (Fig. 2). Among the patients who had had symptoms for five years or less, 7 of 91 patients (8 percent) in the group given omeprazole alone had a response to treatment, as compared with 26 of 98 patients (27 percent) in the group given omeprazole and antibiotics. Among the patients who had had symptoms for more than five years, 3 of 62 patients (5 percent) in the group given omeprazole alone had a response to treatment, as compared with 7 of 56 patients (12 percent) in the group given omeprazole and antibiotics. | *Figure 2.-Percentages of Patients with Resolution of Symptoms in Relation to the Duration of Symptoms **before** Treatment. The resolution of symptoms was defined as a Glasgow Dyspepsia Severity Score of 0 or 1. The numbers of patients in each group are shown above the bars *.**FIGURE OMITTED***

Routine repeated endoscopy was not part of the study protocol. However, nine patients were referred for endoscopy by their primary care physician **during** the year **after** randomization because of severe, persistent dyspepsia. Three of the patients were in the group given omeprazole and antibiotics, and all three had normal findings on endoscopy. In contrast, six of the patients were in the group given omeprazole alone, and four had ulcers. Three of these four patients subsequently received therapy to eradicate *H. pylori*; the symptoms resolved in only one.

Discussion

We found that therapy to eradicate *H. pylori* resulted in more frequent resolution of symptoms than did placebo in patients with nonulcer dyspepsia and documented *H. pylori* infection. Treatment with antibiotics and omeprazole resulted in the resolution of dyspepsia in 21 percent of patients; in contrast, symptoms resolved in 7 percent of patients who were treated with omeprazole alone. These results confirm and extend those of an earlier randomized trial showing symptomatic benefit from eradicating *H. pylori* in nonulcer dyspepsia. (Ref. 22) The beneficial effect of this approach is also reflected by the findings in our study of a trend toward reduced use of prescription-drug therapy for dyspepsia ($P=0.09$) and a shorter duration of such therapy ($P=0.05$) in the group given omeprazole and antibiotics.

We found no evidence that factors associated with duodenal ulcer disease, including smoking, epigastric pain as the predominant symptom, male sex, a high acid output, or a positive test for *H. pylori* CagA, (Ref. 19,23-25) were predictive of the success of treatment with omeprazole and antibiotics. Only the duration of dyspeptic symptoms **before** treatment was predictive of the response. The longer the history of dyspepsia, the less likely the benefit from this therapy. The mechanism of this association is

not clear. It is possible that prolonged, symptomatic *H. pylori*-associated gastritis induces changes that become irreversible, or only slowly reversible, resulting in persistent dyspepsia despite eradication of the infection.

Our study also suggests that patients with *H. pylori* infection and nonulcer dyspepsia are at risk for ulcers. Of the six patients in the group given omeprazole alone who were referred for further evaluation because of severe, persistent dyspepsia, four had peptic ulcers. More ulcers might have been detected if all the patients in this group who had persistent symptoms had undergone follow-up endoscopy. A high incidence of peptic ulcers in patients with *H. pylori* infection and nonulcer dyspepsia has been reported in other studies. In one randomized study, ulcers developed in 14 percent of 50 *H. pylori*-positive patients with nonulcer dyspepsia in the placebo group during the one-year follow-up period. (Ref. 22) In another, similar study, peptic ulcers developed in 21 percent of 41 patients during a mean follow-up period of three years. (Ref. 26)

It is tempting to assume that the subgroup of patients with nonulcer dyspepsia in whom dyspepsia resolved on treatment of *H. pylori* corresponds to the subgroup of patients in the group given omeprazole alone in whom peptic ulcers developed. However, of the three patients in this group in whom ulcers developed and who subsequently underwent treatment for *H. pylori*, only one had resolution of dyspepsia. This finding is consistent with previous reports that the symptomatic response to the eradication of *H. pylori* varies in patients with peptic ulcers. (Ref. 27-29)

Our findings have clinical implications for the treatment of patients with dyspepsia. Previous studies have shown that eradicating *H. pylori* is beneficial in patients with confirmed peptic ulcers; thus, eradication of the infection has been recommended only in such patients. We found that this approach was beneficial in a proportion of *H. pylori*-infected patients with dyspepsia who had no evidence of current or previous ulcers at the time treatment was initiated. Because it is not yet possible to identify prospectively patients whose symptoms will resolve with such treatment, it will be necessary to treat all patients with *H. pylori* infection and nonulcer dyspepsia in order to cure a minority. However, the potential benefit -- curing a chronic disorder with a single course of antibiotic treatment -- would seem to justify this approach.

Our study also has implications for the use of endoscopy in younger patients with uncomplicated dyspepsia. Endoscopy has been used to identify patients with *H. pylori* infection and associated peptic ulcer disease and thus to identify those who should be treated for this infection. Our findings that the eradication of *H. pylori* is also beneficial for patients with no peptic ulcers and that negative endoscopic results do not rule out the possibility of an ulcer in the future raise questions about the appropriateness of endoscopy in determining management. They support a strategy that includes noninvasive testing of patients with dyspepsia for *H. pylori* and eradicating *H. pylori* infection in those with evidence of infection.

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Effect of Eradication of Helicobacter pylori in Patients with Fundic Atrophic Gastritis (Correspondence)

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TEXT

Letter 001

To the Editor: Nonautoimmune atrophic gastritis of the fundus is commonly considered chronic, progressive, and irreversible and is closely associated with gastric carcinoma and endocrine-cell tumors. (Ref. 1,2) Recent investigations support the hypothesis that Helicobacter pylori may have a role in causing this condition. (Ref. 3)

We evaluated the effect of the eradication of H. pylori on histologic findings and secretory-motor gastric functions in five men and seven women (mean +/-SD] age, 48+/-9 years) with nonautoimmune fundic atrophic gastritis and H. pylori infection. Exclusion criteria were the presence of pernicious anemia (identified on the basis of the Schilling test, mean corpuscular volume, vitamin B(sub 12) level, and presence or absence of antibodies against intrinsic factor); the presence of erosive, ulcerative, or malignant lesions on endoscopy; the presence of organic digestive or systemic disease, previous major abdominal surgery, or excess alcohol intake; a history of long-term consumption of acid-suppressing drugs; and previous treatment with vitamin B(sub 12) or use of drugs influencing gastric acid secretion or emptying during the preceding four weeks.

The following investigations were performed, the details of which have been reported elsewhere (Ref. 4): upper gastrointestinal endoscopy (with two antral and four fundic biopsy specimens obtained for histologic studies; one antral and one fundic biopsy specimen for bacteriologic culture; and one antral biopsy specimen for the urease test); histologic evaluation of gastric mucosa (hematoxylin and eosin, periodic acid-Schiff, and Giemsa staining) for the following five characteristics: glandular atrophy, chronic inflammatory-cell infiltration, neutrophilic activity, intestinal metaplasia, and H. pylori density (graded as 0, absent; 1, mild; 2, moderate; or 3, severe); assessment of H. pylori status (on the basis of cultures, histologic studies, and the urease test); measurement of basal and stimulated gastric acid secretion; determination of basal serum gastrin levels; and the gastric-emptying test (performed by means of a scintigraphic technique in which the solid component of a mixed meal is labeled with technetium-99m, with the results expressed as the percentage of gastric contents emptied from the stomach in one minute). The study was approved by the ethics committee of S. Orsola Hospital, and all the patients gave informed consent.

After these investigations had been performed, six patients (group 1) were treated with a 14-day oral course of amoxicillin (500 mg four times a day), metronidazole (250 mg four times a day), and bismuth (120 mg four times a day), and six (group 2) did not receive any treatment. One year later, the base-line investigations were repeated. The patients in group 2 subsequently received the same treatment that had been given to the patients in group 1 and were reevaluated 12 months later.

The results are shown in Table 1. At entry, all the patients had histologic evidence of both fundic atrophic gastritis and H. pylori infection. At one year, all the patients in group 1 were free of H. pylori on the basis of all three studies (culture, histologic evaluation, and the urease test), whereas all the patients in group 2 were still infected. In group 1, both fundic atrophy and acid secretion were improved, as compared with the base-line findings ($P<0.05$). In group 2, no substantial change in either histologic or functional findings was observed at 1 year; conversely, fundic atrophy and acid secretion were improved ($P<0.05$) 12

months later, **after** the bacteria had been eradicated. Gastric emptying was, on average, unaffected by the treatment; however, three patients (two in group 1 and one in group 2) who had delayed gastric emptying at entry had normal emptying **after** the eradication of *H. pylori*.|*Table 1.-Histologic and Functional Findings in 12 Patients with Fundic Atrophic Gastritis and *H. pylori* Infection, **before** and **after** Eradication of the Infection *****TABLE OMITTED****

Our findings suggest that the natural history of atrophic gastritis can be modified by the eradication of *H. pylori*. In view of the relation of this condition to gastric cancer, there is a rationale for eradicating *H. pylori* in patients with this infection and fundic atrophic gastritis.|Antonio Tucci, M.D.Guido Biasco, M.D.Giovanni Francesco Paparo, M.D.University of Bologna40138 Bologna, Italy

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Title: Comparison of invasive and noninvasive methods for the diagnosis and evaluation of eradication of Helicobacter pylori infection in children

Author(s): Yanez P; MadrazodelaGarza A; PerezPerez G; Cabrera L; Munoz O; Torres J (REPRINT)

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Abstract: Background, Acquisition of Helicobacter pylori infection occurs mainly **during** childhood. To study the events associated with H. pylori colonization in children it is important to have reliable diagnostic methods. Our objective was to validate invasive and noninvasive tests for diagnosis of H. pylori infection in children **before** and **after** antimicrobial treatment.

Methods. **Before** treatment, invasive rapid urease test (RUT) culture and histology, as well as the noninvasive carbon-13 urea breath test (C-13-UBT) and serology were validated in 59 children. The gold standard for H. pylori infection was any of three positives of the five tests. **After** antimicrobial treatment culture, histology, and C-13-UBT were validated in 43 children to determine eradication. The gold standard for eradication was negative in all three tests,

Results. For primary diagnosis, RUT was the most sensitive and specific test, followed by C-13-UBT, which performed better than serology, culture, and histology. Concordance tests also showed that RUT and C-13-UBT performed better. For determination of eradication, C-13-UBT and histology were better than culture, which showed poor sensitivity.

Conclusions. RUT performed better for primary diagnosis. However, as endoscopy might not be indicated in most children, C-13-UBT could be the test of choice for diagnosis of H. pylori infection both **before** and **after** eradication treatment, (C) 2000 IMSS. Published by Elsevier Science Inc.

Descriptors--Author Keywords: urease test ; histology ; serology ; gastrointestinal tract ; gold standard

Identifiers--KeyWord Plus(R): C-13-UREA BREATH TEST; RECURRENT ABDOMINAL-PAIN; **IMMUNE** -RESPONSE; CAMPYLOBACTER-PYLORIDIS; GASTRITIS; EPIDEMIOLOGY; TRANSMISSION; PREVALENCE; CHILDHOOD; COMMUNITY

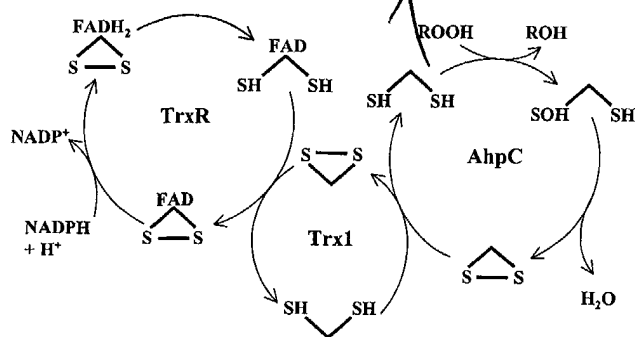


FIG. 9. Pathway for transfer of reducing equivalents from NADPH to hydroperoxide in the alkyl hydroperoxide reductase system from *H. pylori*. Note that the enzyme species shown do not necessarily represent actual catalytic intermediates. FAD, flavin adenine dinucleotide.

detectable enzyme-substrate complexes. This ping-pong mechanism has also been observed for the distantly related glutathione peroxidases (23, 68). The higher rate of enzyme-peroxide interaction for glutathione peroxidase ($\sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$) has been attributed to the unique reactivity of the selenocysteine at the active site (64), although at 10^5 to $10^6 \text{ M}^{-1} \text{ s}^{-1}$, the rate of peroxide reduction is still quite high and indicative of an important role for *H. pylori* AhpC in cellular peroxide metabolism. Using k_1' to characterize the protein-peroxide interaction, our experiments demonstrated essentially no specificity when AhpC was tested with a wide variety of small, bulky, aromatic, or lipid hydroperoxide substrates, as was true of the *C. fasciculata* peroxidase (46). Differential reactivities toward particular hydroperoxide substrates have been reported for some other Prx enzymes based on less quantitative analyses (9, 33, 34). In addition, peroxynitrite (OONO^-) has recently been shown to be a substrate for *H. pylori* AhpC, with the rate of decomposition occurring at a second-order rate constant of $1.21 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (7). These results are all consistent with a minimal binding site on AhpC for hydroperoxides (and peroxynitrite) consisting of little more than the catalytic residue (Cys49) at the active site.

On the basis of results reported here and mechanisms outlined for other 2-Cys AhpC homologues (10, 51), electrons from NADPH proceed along the path outlined in Fig. 9 for the reduction of peroxides to alcohols. This scheme is highly analogous to that for electron transfer through the *S. typhimurium* AhpC system, except that TrxR and Trx1 replace AhpF in the *H. pylori* system. Nonetheless, one tightly bound flavin and three disulfide redox centers mediate electron transfer from pyridine nucleotide to peroxide in both cases.

Little information exists on potential redox or iron regulation of *H. pylori* AhpC expression, although in other studies of TrxR and Trx1 from *H. pylori*, Windle et al. (74) observed that Trx1 expression dramatically increased under conditions of oxidative stress; Trx1 was therefore classified as a stress response element in *H. pylori*. While the proximal location of *trxR* and *trx1* in the chromosome could provide the bacterium with a mechanism for a coordinated response eliciting expression of both proteins, an increase in TrxR expression did not accompany increased Trx1 expression under oxidative stress conditions (74). Interestingly, reductase activity of the TrxR-Trx1 reductase system was also reportedly present in the media of

culture supernatants and could be available to support extracellular peroxide reduction by AhpC if the latter protein is also exported.

In conclusion, this is the first report of a Trx-dependent alkyl hydroperoxide system from a gastric pathogen. To our knowledge, AhpC from *H. pylori* is only the second known bacterial AhpC to be demonstrated experimentally to require the Trx-reducing system for reduction of the oxidized AhpC active site. An essential role for AhpC in *H. pylori* has been established with our *ahpC* mutagenesis experiments, and this is, to our knowledge, the only case in which an *ahpC* locus has been shown to be required for viability. The presence of this essential Trx-dependent peroxidase in *H. pylori* suggests important roles for TrxR, Trx1, and AhpC in the removal of alkyl hydroperoxides to protect against oxidative stress and in the preservation of the microaerobic environment required for *H. pylori* viability.


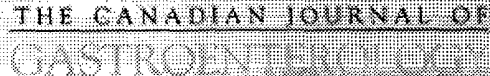
ACKNOWLEDGMENTS

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Original Article

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Utility of serology in determining *Helicobacter pylori* eradication after therapy

CA Fallone, VG Loo, AN Barkun

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OBJECTIVE: To determine the usefulness of four serological tests in confirming cure of *H pylori* infection before the previously reported six-month post-treatment delay.

PATIENTS AND METHODS: As part of a prospective, blinded, controlled trial, in which patients with duodenal ulcers were randomized to receive different combinations of antibiotics, serum samples were obtained in 89 patients before treatment, as well as on several occasions after treatment. Antibody titres were determined by ELISA with Bio-Rad immunoglobulin (Ig) A, Bio-Rad IgG, Pyloriset EIA-A for IgA and Pyloriset EIA-G for IgG. Eradication was confirmed with antral biopsy three months after therapy.

RESULTS: The percentage drop in titre following treatment was significantly larger for the group of patients who were treated successfully with all four kits. Optimal cut-offs for identifying successful therapy were determined, and accuracy improved as the interval between testing and therapy was prolonged. Six months after therapy, the IgG test from Bio-Rad achieved 100% sensitivity and 80% specificity, and that from Pyloriset achieved 88% sensitivity and 100% specificity. At three months, however, test performance was quite good, with 90% sensitivity and 80% specificity when using a Pyloriset IgA titre drop of 20% or greater to predict successful eradication.

CONCLUSION: Serology is a simple, easily available, noninvasive method that exhibits good positive predictive value in the confirmation of successful cure of *H pylori* infection three or six months after treatment.



Key Words: *Helicobacter pylori*, Immunoglobulin A, Immunoglobulin G, Serology, Treatment



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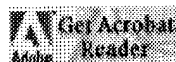


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Utilité des tests sérologiques dans l'évaluation du traitement d'éradication contre *Helicobacter pylori*

DONNÉES DE DÉPART : La sérologie est une méthode simple et non effractive de dépistage de *H. pylori*.

OBJECTIFS : Déterminer l'utilité de quatre tests sérologiques pour confirmer l'éradication de *H. pylori*, avant le délai post-thérapeutique de six mois recommandé autrefois.

PATIENTS ET MÉTHODES : Dans le cadre d'un essai contrôlé, prospectif, mené à l'insu, regroupant des patients atteints d'ulcères duodénaux, randomisés afin de recevoir différentes associations d'antibiotiques, des échantillons sériques ont été prélevés chez 89 patients avant le traitement et à plusieurs occasions après ce dernier. Les titres d'anticorps ont été mesurés par ELISA avec l'immunoglobuline Bio-Rad A (Ig), Bio-Rad IgG, Pyloriset EIA-A pour IgA et Pyloriset EIA-G pour l'IgG. L'éradication a été confirmée par biopsie antrale trois mois après le traitement.

RÉSULTATS : En pourcentage, la baisse des titres après le traitement a été significativement plus marquée dans le groupe de patients traités avec succès au moyen des quatre nécessaires. Les seuils idéaux pour l'identification de la réussite du traitement ont été déterminés et le degré de précision a été amélioré proportionnellement avec la durée de l'intervalle entre le test et le traitement. Six mois après le traitement, le test à l'IgG de Bio-Rad a manifesté une sensibilité de 100 % et une spécificité de 80 % et le Pyloriset a manifesté une sensibilité de 88 % et une spécificité de 100 %. Après trois mois, toutefois, le rendement a été assez bon, avec 90 % de sensibilité et 80 % de spécificité en se fiant sur une baisse du titre de l'ordre de 20 % ou plus avec le Pyloriset IgA pour prédire la réussite de l'éradication.

CONCLUSION : L'analyse sérologique est une méthode simple, accessible et non effractive qui donne une valeur prédictive positive appréciable pour la confirmation de la réussite du traitement d'éradication de *H. pylori* trois ou six mois après le traitement.



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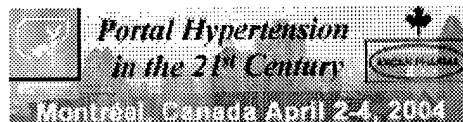
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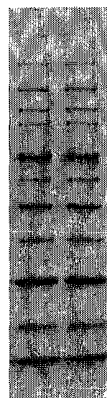
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PROTEIN STANDARDS (shown above) are available for purchase:

Novel β -Secretase Cleavage of β -Amyloid Precursor Protein in the Endoplasmic Reticulum/Intermediate Compartment of NT2N Cells

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Abstract. Previous studies have demonstrated that NT2N neurons derived from a human embryonal carcinoma cell line (NT2) constitutively process the endogenous wild-type β -amyloid precursor protein (APP) to amyloid β peptide in an intracellular compartment. These studies indicate that other proteolytic fragments generated by intracellular processing must also be present in these cells. Here we show that the NH_2 -terminal fragment of APP generated by β -secretase cleavage (APP_β) is indeed produced from the endogenous full length APP (APP_{FL}). Pulse-chase studies demonstrated a precursor-product relationship between

APP_{FL} and APP_β as well as intracellular and secreted APP_β fragments. In addition, trypsin digestion of intact NT2N cells at 4°C did not abolish APP_β recovered from the cell lysates. Furthermore, the production of intracellular APP_β from wild-type APP appears to be a unique characteristic of postmitotic neurons, since intracellular APP_β was not detected in several non-neuronal cell lines. Significantly, production of APP_β occurred even when APP was retained in the ER/intermediate compartment by inhibition with brefeldin A, incubation at 15°C, or by expression of exogenous APP bearing the dilysine ER retrieval motif.

AMYLOID β ($\text{A}\beta$)¹ peptides are the building blocks of the amyloid fibrils found in neuritic plaques and vascular deposits that accumulate in the brains of patients with Alzheimer's disease (AD; Selkoe, 1994). $\text{A}\beta$ is derived from proteolytic processing of one or more isoforms of the amyloid precursor protein (APP; Kang et al., 1987). APP isoforms are alternatively spliced type I transmembrane glycoproteins that are encoded by a single gene on human chromosome 21 (Kang et al., 1987; St. George-Hyslop et al., 1987). The 39–43-amino acid-long $\text{A}\beta$ sequence begins in the ectodomain of APP and extends into the transmembrane region (see Fig. 1). Of the three major $\text{A}\beta$ -containing isoforms encoded by the APP gene (i.e., APP695, APP751, and APP770; Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988), APP695 is expressed almost exclusively by neurons of the central and peripheral nervous systems (Golde et al., 1990; Kang and Müller-Hill, 1990; Arai et al., 1991).

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1. *Abbreviations used in this paper:* $\text{A}\beta$, amyloid β ; AD, Alzheimer's disease; APP, amyloid precursor protein; BFA, brefeldin A; CNS, central nervous system; Endo H, endoglycosidase H; IC, intermediate compartment; Nglyc F, N-glycosidase F; SFV, Semliki Forest virus.

Newly synthesized APP matures in the endoplasmic reticulum and the Golgi apparatus, acquiring *N*- and *O*-linked carbohydrates, tyrosine sulfates (Weidemann et al., 1989; Oltersdorf et al., 1990), and phosphates (Oltersdorf et al., 1990; Suzuki et al., 1992; Knops et al., 1993). Several pathways of APP metabolism have been described in cultured cells, and evidence suggests that the relative importance of each pathway depends on the cell type. For example, non-neuronal cells preferentially process APP by the α -secretase pathway, which cleaves APP within the $\text{A}\beta$ sequence, thereby precluding the formation of $\text{A}\beta$ (Esch et al., 1990; Sisodia et al., 1990). The putative α -secretase enzyme(s) is active at or near the cell surface, causing the NH_2 -terminal fragment (APP_α) to be quickly secreted. In contrast, neuronal cells process a much larger portion of APP by the β -secretase pathway(s), which generate intact $\text{A}\beta$ by the combined activity of two enzyme classes. The β -secretase(s) cleaves APP at the NH_2 terminus of the $\text{A}\beta$ domain releasing a distinct NH_2 -terminal fragment (APP_β). In addition, the γ -secretase(s) cleaves APP at alternative sites of the COOH terminus, generating species of $\text{A}\beta$ that are either 40 ($\text{A}\beta_{40}$) or 42 amino acids long ($\text{A}\beta_{42}$; Seubert et al., 1993; Suzuki et al., 1994; Turner et al., 1996).

Although the identities of the putative α -, β -, and γ -secretases remain speculative, and the precise subcellular localization of their activity is poorly understood, in vitro studies have suggested the existence of at least two

β -secretase pathways. In the endosomal/lysosomal pathway, APP targeted to the cell surface is endocytosed and delivered to endosomes and lysosomes where β - and γ -cleavages can occur (Golde et al., 1992; Haass et al., 1992a; Nordstedt et al., 1993; Koo and Squazzo, 1994; Lai et al., 1995; Perez et al., 1996). The alternative β -secretory pathway is predicted to generate A β in Golgi-derived vesicles, most likely secretory vesicles, before secretion (Haass et al., 1995a; Higaki et al., 1995; Perez et al., 1996; Thinakaran et al., 1996b). Whether these pathways operate in the same or different cell types is not known, nor is the biological importance of each pathway for the production of A β in vivo understood.

Recently, we showed that both A β_{40} and A β_{42} are produced intracellularly from endogenous wild-type APP695 by cultured postmitotic central nervous system (CNS) neuronal cells (NT2N) that are induced to differentiate from a human teratocarcinoma cell line (NT2) by treatment with retinoic acid (Pleasure et al., 1992; Pleasure and Lee, 1993; Wertkin et al., 1993; Turner et al., 1996). To date, the human-derived NT2N neuron is the only cell line documented to generate intracellular A β_{40} and A β_{42} before their eventual release into the medium (Turner et al., 1996). Because neurons are the cell type most adversely affected by AD, the NT2N neurons represent a unique system for the study of intracellular β -secretase pathways in a human neuronal model. An essential first step in the analysis of such pathways is the identification of the proteolytic fragments that are the products of these cleavages. We report here that in addition to A β_{40} and A β_{42} , the NH₂-terminal fragment generated by β cleavage (i.e., APP β) is produced intracellularly in NT2N neurons before secretion. More significantly, we demonstrate that novel β -secretase activity occurs in the ER/intermediate compartment (IC) of neuronal cells using inhibition with Brefeldin A (BFA), incubation at 15°C, and expression of exogenous APP bearing the dilysine ER-retrieval motif.

Materials and Methods

Cell Culture

NT2 cells derived from a human embryonal carcinoma cell line (Ntera 2/cl.D1) were grown and passaged twice weekly in Opti-Mem (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% FBS and penicillin/streptomycin (P/S) as described previously (Pleasure et al., 1992; Pleasure and Lee, 1993). To begin differentiation, 2.5×10^6 cells were seeded in a 75-cm² (T75) flask and fed with DME HG (Life Technologies, Inc.) containing 10 μ M retinoic acid, 10% FBS, and P/S twice weekly for 5 wk. The cells in a single T75 flask were then replated at a lower density in 2×225 cm² (T225) flasks for 10 d (Replate 1 cells). Greater than 99% pure NT2N neurons were then obtained by enzymatic treatment and mechanical dislodgment of Replate 1 cells and replated at a density of 6×10^6 cells/10-cm dish previously coated with polylysine and Matrigel (Pleasure et al., 1992). The NT2N neurons were maintained in medium consisting of one part conditioned medium and one part DME HG containing 10% FBS and P/S. For experiments involving the incubation of NT2N neurons at 15°C for 16 h, regular medium containing DME HG and 10% FBS was replaced by DME HG containing 25 mM Hepes, 10% FBS, and P/S. Cultures of NT2N neurons were used for experiments when they were between 3 to 4 wk old. CHO695 cells, a gift from Dr. S. Sisodia (Johns Hopkins University School of Medicine, Baltimore, MD), were grown and passaged three times per week in α -MEM (Life Technologies, Inc.) supplemented with 10% FBS and P/S. M17 cells were grown and passaged once per week in Opti-Mem (Life Technologies, Inc.) containing 10% iron-enriched calf serum and P/S.

Metabolic Labeling, Gel Electrophoresis, Immunoblotting, and Quantitation

Cultured NT2N neurons were starved in methionine-free DME HG (Life Technologies, Inc.) for 30 min before incubation in fresh, methionine-free DME HG containing 0.5 mCi/ml of [³⁵S]methionine (sp act 1,000 Ci/mmol; NEN-Du Pont, Boston, MA). For steady-state labeling studies, NT2N neurons were labeled with [³⁵S]methionine continuously for 16 h. For pulse-chase studies, cells were labeled with [³⁵S]methionine for 1 h, washed twice with methionine-containing DME, and then chased in the same medium for 0 to 24 h. APP_{FL}, APP α , and APP β were separated on 7.5% Laemmli SDS-PAGE gels, and A β and p3 were separated on 10/16.5% step-gradient Tris-tricine gels. These gels were either stained with Coomassie brilliant blue R (Pierce, Rockford, IL) and dried or transferred to nitrocellulose membranes and dried before exposure on PhosphorImager plates (Molecular Dynamics, Sunnyvale, CA) for 3–5 d. The nitrocellulose replicas containing the immunoprecipitates were further probed with different antibodies, as described previously (Wertkin et al., 1993). Quantitation of bands in the autoradiogram was performed using the ImageQuant software (Molecular Dynamics) as described previously (Turner et al., 1996). Radiolabeled proteins in SDS-PAGE gels and nitrocellulose replicas were also analyzed by standard autoradiographic methods. All experiments were repeated between three to six times.

Sample Preparation and Serial Immunoprecipitations

Cell lysates were prepared as described elsewhere (Golde et al., 1992). Protein concentration was determined by the bicinchoninic acid procedure (Pierce). Media were centrifuged at 100,000 g for 1 h at 4°C before immunoprecipitation. Both cell lysates and media were precleared with protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) in RIPA for 1 h at 4°C. After recentrifugation at 15,000 g for 1 min, the supernatants were rocked overnight at 4°C with fresh protein A-Sepharose and the appropriate primary antibody. After collecting the immunoprecipitates by recentrifugation at 15,000 g for 1 min, the supernatants were used in a second round of immunoprecipitation with fresh protein A-Sepharose and a different primary antibody.

Trypsin Treatment of NT2N Neurons

NT2N neurons were metabolically labeled with 0.5 mCi/ml [³⁵S]methionine for 16 h, as described above. After rinsing the cultures twice with PBS, the NT2N neurons were incubated on ice for 20 min with PBS, with 10 μ g/ml of trypsin in PBS alone (Life Technologies, Inc.), or with 10 μ g/ml trypsin and 0.1% Triton X-100 in PBS. After this treatment, trypsin was inactivated by the addition of 100 μ g/ml soybean trypsin inhibitor. The cells were then washed with PBS, scraped into cell lysis buffer, and processed for immunoprecipitation, as described above.

BFA Treatment of NT2N Neurons and Deglycosylation of Immunoprecipitated APP β

NT2N neurons were pretreated with 20 μ g/ml of BFA for 1 h before the addition of 0.5 mCi/ml of [³⁵S]methionine to the cultures for 16 h in the absence or presence of BFA. The cell lysates and media were processed for immunoprecipitation as described above. For deglycosylation of APP β , the immunoprecipitates containing APP β were washed twice in sodium phosphate buffer (20 mmol/liter, pH 7.2) and boiled for 2 min in 10 μ l of 1% SDS. The samples were then boiled for an additional 2 min after adding 90 μ l of the sodium phosphate buffer with sodium azide (10 mmol/liter), EDTA (50 mmol/liter), and *n*-Octylglucoside (0.5% wt/vol). After the denaturation step as described, deglycosylation was initiated by the addition of 2 mU neuraminidase (Arthrobacter; Boehringer Mannheim, Indianapolis, IN), 2.5 mU *O*-Glycosidase (Boehringer Mannheim), and 0.4 U *N*-Glycosidase F (Boehringer Mannheim). The samples were then incubated at 37°C for 18 h, and deglycosylated APP β was run on 7.5% SDS-PAGE gels as described above. For endoglycosidase H (Endo H) sensitivity test, cell lysates and media were immunoprecipitated with Karen as described. The immunoprecipitates were then recovered in 100 μ l 60 mM phosphate buffer, pH 5.7, with 1% SDS. The samples were then split in half (50 μ l each) and incubated with 4 μ l Endo H (Boehringer Mannheim) or vehicle at 37°C for 18 h. The samples were then run on 7.5% SDS-PAGE gels as described above.

Antibodies for Immunoprecipitation and Immunoblotting

The antibodies used in this study and their epitope specificities are summarized in Fig. 1. Briefly, Karen is a goat polyclonal antiserum raised to the large, secreted NH₂-terminal fragment of APP, and antibody 53 is a rabbit polyclonal antiserum raised to a synthetic peptide corresponding to the amino acid sequence SEVKM. Antibody 53 binds specifically to the free COOH terminus of APP β (Howland et al., 1995). Antibody 369W is a rabbit polyclonal antiserum raised to a synthetic peptide corresponding to the last 45 amino acid residues at the COOH terminus of APP and was generously donated by Dr. Sam Gandy (Cornell University School of Medicine, New York, NY). Also used in this study were three mAbs to A β that are specific for residues 1–17 (6E10; Kim et al., 1988, residues 1–10 (Ban50; Suzuki et al., 1994), and residues 18–25 (4G8; Kim et al., 1988).

Preparation of SFV-bearing pSFV-1(APP695) and pSFV-1(APP695 Δ KK)

The dilysine motif was introduced into APP695 by standard PCR site-directed mutagenesis of pSFV-1(APP695) using primers 5'-CGAAAAC-CACCGTGGAGCTCC TT-3' and 5'-TTAACCCGGGCTAGTTCT-GCTTCTTCTCAAAGAAGCTTGT-3'. The mutation-containing PCR fragment was isolated by digestion with BsmI and XmaI and then ligated into pSFV(APP695) to yield pSFV(APP695 Δ KK). All pSFV-1 constructs, including a pSFV helper plasmid with SFV structural genes, were linearized by digestion with SpeI and then used as a template for RNA synthesis with SP6 RNA polymerase. Coelectroporation of RNA from the expression and helper plasmids into BHK cells yielded an infectious, replication-defective virus that was harvested 24 h later (Liljestrom and Garoff, 1991). Accurate determination of viral stock titers was made as described elsewhere (Cook et al., 1996). For all infection experiments, $\sim 1 \times 10^6$ NT2N neurons per 35-mm dish were infected in serum-free medium at a multiplicity of infection (MOI) of 7–10. When called for, 20 μ g/ml BFA was added after the completion of the infection step.

Results

NT2N Neurons Exhibit Intracellular β -Secretase Activity

Our previous studies have demonstrated that NT2N cells produce intracellular A β (Wertkin et al., 1993; Turner et al., 1996). To determine if intracellular APP β (Fig. 1) can also be recovered from these cells, samples of cell lysate were immunoprecipitated with Karen (an antiserum raised to the NH₂-terminal region of APP). Then, the presence of APP β in the immunoprecipitate was determined by immunoblot analysis using 53 (a polyclonal antibody specific for the free COOH terminus of APP β). We found that 53 de-

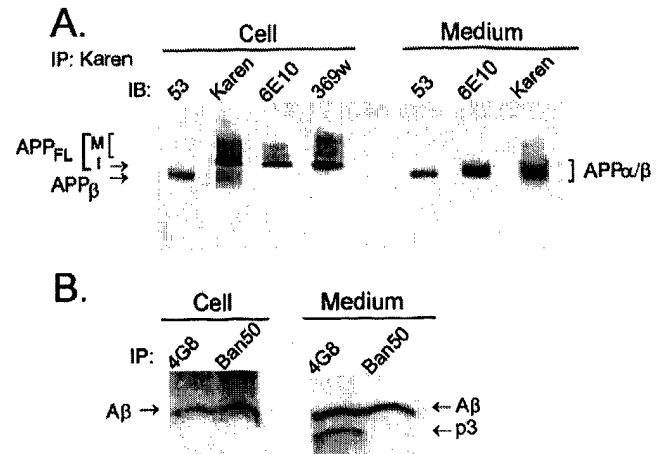


Figure 2. NT2N neurons produce intracellular APP β and A β . To demonstrate the presence of APP β , samples of cell lysate and medium were collected from NT2N cultures and processed for immunoprecipitation (IP) with Karen, a polyclonal antibody that recognizes epitopes within the large ectodomain of APP. The presence of APP β , APP α , APP α/β , and APP $_{FL}$ was detected by immunoblotting (IB) with the corresponding antibodies (A). To show that A β but not p3 is produced intracellularly, NT2N neurons were radiolabeled with [³⁵S]methionine for 16 h. The cell lysate and the medium were then processed for immunoprecipitation with 4G8, a mAb that binds to both A β and p3, or Ban50, a mAb that recognizes only A β (B). Immunoprecipitates of A β and p3 were separated by electrophoresis in 10/16.5% step-gradient Tris-tricine gels. M, mature APP $_{FL}$; I, immature APP $_{FL}$.

fects a single band of ~ 95 kD (Fig. 2 a). That this 95-kD APP fragment is indeed APP β , cleaved at the β -secretase site, was further substantiated by (a) the inability of 369W, an antibody specific for the COOH terminus of APP, to recognize this fragment; (b) the inability of 6E10, an antibody specific for the first 10 amino acid residues of A β , to detect this fragment; (c) the binding of Karen, an antibody that recognizes all APP species, to this fragment; (d) the fact that this intracellular APP fragment is ~ 11 – 12 kD smaller than APP $_{FL}$ (Fig. 2 a); and (e) the detection of the same 95-kD APP fragment using a different antibody specific for APP β (i.e., 192; Seubert et al., 1992; and data not shown). To determine if APP β is secreted, media from

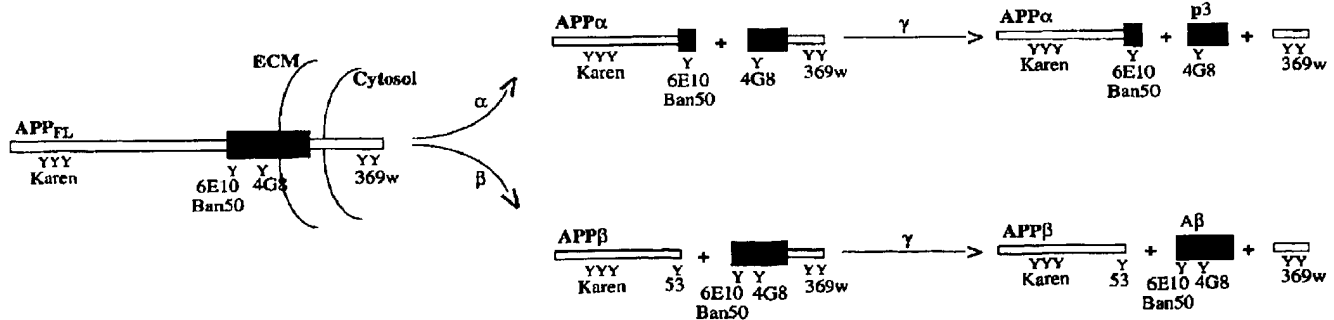


Figure 1. Proteolytic processing of APP $_{FL}$. The diagram depicts APP fragments generated by both the α - and β -secretase pathways. A large, secreted ectodomain called APP α is generated by the putative α -secretase(s) that cleaves APP $_{FL}$ within the A β domain. A second cleavage by the γ -secretase(s) releases a subfragment of A β known as p3. Alternative cleavage by the β -secretase(s) generates a similarly large ectodomain fragment known as APP β . After the subsequent γ -secretase cleavage, A β is released. This schematic also shows the epitope location of the antibodies used in this study to identify the different proteolytic fragments.

NT2N neurons were again immunoprecipitated with Karen and subsequently immunoblotted with various antibodies (Fig. 2 *a*). We found that APP β was readily detected in the media of NT2N neurons and that it comigrated with APP β recovered from the cell lysates. However, as expected, APP β migrated slightly faster than the product of α -secretase cleavage (APP α), which was also recovered from the media.

The detection of intracellular APP β and A β in NT2N neurons is consistent with our view that both β - and γ -secretase activities occur in an intracellular compartment. The absence of intracellular APP α , however, suggests that the majority or all of the α -secretase activity occurs at a different site. To further confirm that the β -secretase pathway, but not the α -secretase pathway, occurs inside these cells, we examined the cell lysate of NT2N neurons for the products of these respective pathways: A β , which is generated by β - and γ -secretase cleavages; and p3, a product of α - and γ -secretase cleavages. To do this, we immunoprecipitated the cell lysates of metabolically labeled NT2N neurons with mAbs that can distinguish between these peptides: 4G8 recognizes both A β and p3; Ban50, however, binds only to A β and not p3 (Fig. 2 *b*). Our data clearly demonstrate that A β , but not p3, is produced intracellularly. The p3 fragment was not detected in cell lysates even after prolonged exposure of the film. By contrast, both A β and p3 were readily recovered from the media. This observation supports previous findings that the α -secretase pathway occurs at or near the plasma membrane (Haass et al., 1992a, 1995b; Sisodia, 1992).

To determine if the recovery of APP β from the cell lysates reflects its intracellular origin or its association with the cell surface, we treated cultures of NT2N neurons with trypsin at 4°C. Under such conditions, cell surface-associated but not intracellular APP β should be proteolyzed. Fig. 3 shows that a similar amount of APP β was recovered from NT2N neurons regardless of trypsin treatment (Fig. 3, compare lanes 1 and 2). By contrast, when the NT2N neurons were treated with trypsin and 0.1% Triton X-100, intracellular APP β was completely eliminated (Fig. 3, lane

IP: 53

Trypsin: - + +
Tx-100: - - +

APP β →

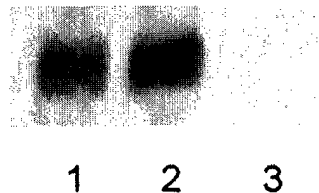


Figure 3. APP β is produced intracellularly in NT2N neurons. Culture dishes containing >99% pure NT2N cells were metabolically labeled with [³⁵S]methionine for 16 h. Cells were rinsed twice with PBS and then incubated on ice for 20 min with PBS alone (lane 1), with 10 μ g/ml trypsin (lane 2), or with 10 μ g/ml trypsin and 0.1% Triton X-100 (lane 3). The cells were processed for immunoprecipitation with the anti-APP β antibody 53, as described in Materials and Methods.

3). This experiment provides evidence that the APP β recovered from the NT2N cell lysate is indeed produced in an intracellular compartment.

Intracellular APP β Derived from Wild-Type APP Is Detected Only in Cells with a CNS Phenotype

To determine if other cell types are capable of producing intracellular APP β , the following cell lines were included in this study for comparison: (a) retinoic acid-naïve NT2 cells, the undifferentiated precursors of the NT2N neurons that express high levels of the APP751 and APP770 isoforms; (b) Chinese hamster ovary (CHO) cells stably transfected with APP695; and (c) human M17 neuroblastoma cells. Approximately 800 μ g of total protein collected in the cell lysates of each cell type was first immunoprecipitated with Karen and then immunoblotted with either antibody 53 to detect APP β (Fig. 4 *a*) or Karen to detect all forms of APP (Fig. 4 *b*). We found that while all four cell types synthesized similar amounts of APP, the NT2N neuron was the only cell type capable of producing detectable levels of intracellular APP β (Fig. 4 *a*). However, both NT2N neurons and stably transfected CHO cells expressing APP695, but neither NT2 cells nor the M17 neuroblastoma cells, secreted APP β , raising the possibility that secretion of APP β may be isoform specific. While our data does not preclude low levels of intracellular β -secretase activity or faster rate of APP β secretion in these cell lines, the evidence clearly indicates that the fraction of APP processed by β -secretase(s) as well as the subcellular site(s) of this activity may be strongly cell-type dependent.

NT2N Neurons Produce Intracellular APP β Before Secretion

The experiments shown in Figs. 2–4 demonstrated that intracellular APP β can be detected in NT2N neurons. These data suggest that APP β may be generated inside the cell before secretion. To demonstrate unequivocally that a precursor-product relationship exists between intracellular and secreted APP β , we adopted the following approaches. In

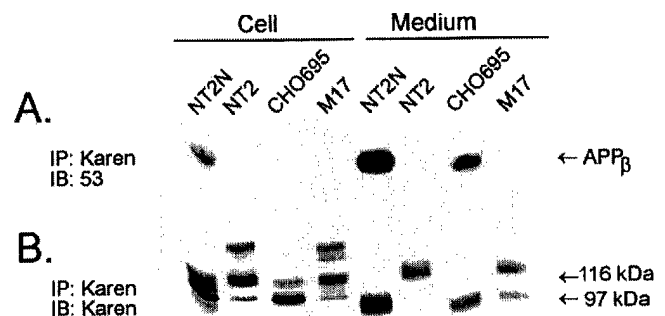


Figure 4. Intracellular APP β is observed only in NT2N neurons. Samples of cell lysate and medium collected from cultures of NT2N, NT2, M17, and CHO cells stably expressing APP695 (CHO695) were processed for immunoprecipitation with the antibody Karen. The immunoprecipitates were separated by SDS-PAGE gels and transferred onto nitrocellulose replicas. APP β present in the cell lysates and the media were detected by immunoblotting with the anti-APP β antibody 53 (*A*). After stripping the nitrocellulose replica in *A* with 0.1% SDS, the blot was reprobed with Karen to detect all APP ectodomain species (*B*).

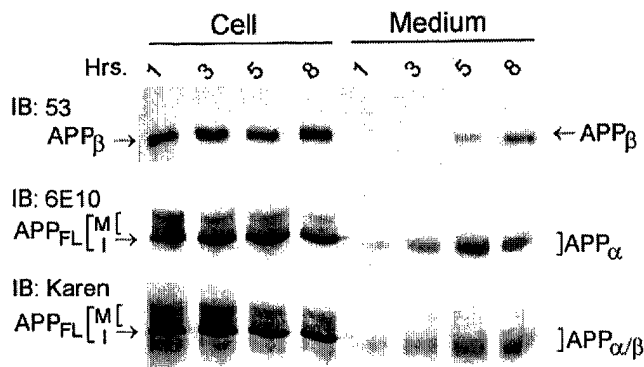


Figure 5. NT2N neurons produce intracellular APP β before secretion. Cultures of NT2N neurons were washed and fresh medium was replenished before measuring the amount of intracellular and secreted APP β over an 8-h period. Cell lysate and medium collected at the times indicated were immunoprecipitated with Karen. The immunoprecipitates were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. APP β was identified in immunoblots using the antibody 53. APP α was detected using the antibody 6E10. APP $_{FL}$ and APP α/β were recognized by Karen.

our first approach, NT2N neurons were washed with fresh medium, and then the amount of intracellular as well as secreted APP β and APP α were measured over an 8-h period. This was accomplished by immunoprecipitation of cell lysates and media with Karen followed by immunoblotting with either antibody 53 (for APP β) or 6E10 (for APP α). As shown in Fig. 5, secreted APP β was first detected in 3 to 5 h, and its accumulation in the medium continued over the 8-h incubation period. By contrast, APP α was detected in 1 h, suggesting that APP α is produced at a faster rate than APP β . As seen with APP β , APP α accu-

mulated in the conditioned media over time. Finally, our data also show that intracellular APP β is produced constitutively, since a steady state level of APP β is recovered from NT2N cell lysates prepared from parallel cultures over a period of 8 h (Fig. 5). These findings are consistent with the idea of APP β being generated inside NT2N neurons before secretion.

We next employed a pulse-chase paradigm to study more rigorously the temporal relationship between intracellular and secreted APP β . To this end, NT2N cultures were pulsed with [35 S]methionine for 1 h and then chased for different lengths of time (Fig. 6). We found that after 1 h of chase time, full length APP (APP $_{FL}$) immunoprecipitated from the cell lysate began to decline, while the intracellular level of APP β continued to increase until 4 h, after which it also declined (Fig. 6, *a* and *c*). This lag in maximum production of intracellular, radiolabeled APP β supports the idea that APP β is produced intracellularly from APP $_{FL}$ by β -secretase cleavage. Finally, the 1-h delay in the secretion of APP β into the medium as well as the accumulation of this fragment with increasing chase time supports a temporal relationship between APP β that is produced intracellularly and APP β that is secreted into the medium (Fig. 6, *b* and *d*). Therefore, we conclude that APP β is produced in an intracellular compartment in NT2N neurons before secretion.

Intracellular β -Cleavage in NT2N Neurons Occurs in a pre-Golgi Compartment

Since APP β is produced in an intracellular compartment in NT2N neurons, we sought to identify the subcellular site(s) of β -secretase cleavage. Therefore, NT2N neurons were metabolically labeled with [35 S]methionine in the presence or absence of 20 μ g/ml BFA (Fig. 7). BFA is a pharmacological agent that causes a redistribution of the Golgi

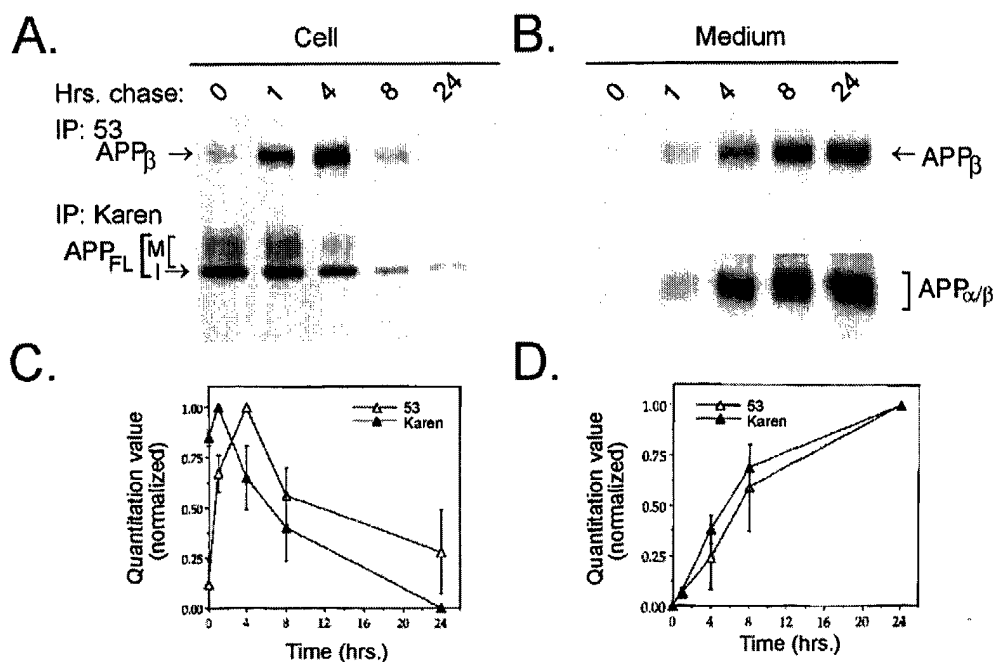


Figure 6. Pulse-chase labeling demonstrates that intracellular APP β is produced in an intracellular compartment before secretion in NT2N neurons. NT2N neurons were pulse labeled with [35 S]methionine for 1 h and chased for 0, 1, 4, 8, and 24 h. Radiolabeled cell lysates (*A*) or media (*B*) were immunoprecipitated sequentially with antibody 53 (for APP β) followed by Karen (for APP $_{FL}$ in the cell lysates and APP α/β in the media). Radiolabeled immunoprecipitates were used to expose PhosphorImager plates (72 h) or X-ray film (3 wk) for visualization. *C* and *D* summarize the quantitation of experiments shown in *A* and *B*. Counts from three different experiments were normalized to percentage of maximum and plotted as shown (mean \pm standard error).

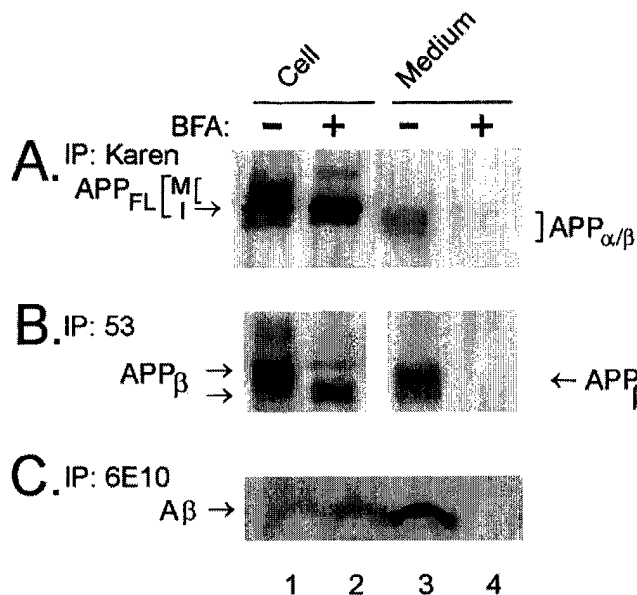


Figure 7. Intracellular β and γ cleavages occur in a pre-Golgi compartment in NT2N neurons. Cultures of NT2N cells were first preincubated with 20 $\mu\text{g/ml}$ BFA for 1 h before radiolabeling with [^{35}S]methionine for 16 h in the continuous presence of 20 $\mu\text{g/ml}$ BFA. Control cultures were processed similarly, except that BFA was absent in the medium. Radiolabeled proteins from BFA-treated and untreated cell lysates and media were immunoprecipitated with Karen (for APP_{FL} in the cell lysates and $\text{APP}_{\alpha/\beta}$ in the media as shown in *A*), with antibody 53 (for APP_{β} in *B*), and with the mAb 6E10 (for $\text{A}\beta$ in *C*). Note that APP_{β} and $\text{A}\beta$ were recovered in the cell lysate but not in the medium of BFA-treated cells. (*M*, mature APP_{FL} ; *I*, immature APP_{FL}).

into the ER (Doms et al., 1989; Lippincott-Schwartz, 1989; Pelham, 1991). In the absence of BFA, APP_{FL} , APP_{β} , and $\text{A}\beta$ were recovered from the cell lysates, while APP_{α} , APP_{β} , and $\text{A}\beta$ were detected in the media of NT2N neurons (Fig. 7, *a-c*, lanes 1 and 3). Surprisingly, in the presence of BFA, not only APP_{FL} but also APP_{β} and $\text{A}\beta$ continued to be recovered from NT2N cell lysates (Fig. 7, *a-c*, lane 2). The effectiveness of BFA was verified by the fact that the secretion of APP_{α} , APP_{β} , and $\text{A}\beta$ into the medium was completely abolished in its presence (Fig. 7, *a-c*, lane 4). Furthermore, we found that APP_{β} recovered from BFA-treated cells (Fig. 7 *b*, lane 2) migrate with an accelerated electrophoretic mobility compared to APP_{β} from nontreated cells (Fig. 7 *b*, lane 1), suggesting that this fragment may have been derived from immature APP. Indeed, the faster mobility of mature APP_{FL} in the presence of BFA (Fig. 7 *a*, compare *M* of lanes 1 and 2) indicates that this agent blocks APP from acquiring at least some of the posttranslational modifications. Thus, $\text{A}\beta$ may be generated from immature as well as mature forms of APP.

We sought next to determine if incomplete maturation of APP is indeed the cause of the shift in electrophoretic mobility of the APP_{β} fragment generated in the presence of BFA. Therefore, NT2N cells were metabolically labeled with [^{35}S]methionine in the presence or absence of BFA, and APP_{β} immunoprecipitated from the cell lysate was incubated with *N*-glycosidase F (Nglyc F), an enzyme that removes N-linked carbohydrate chains. As shown, APP_{β}

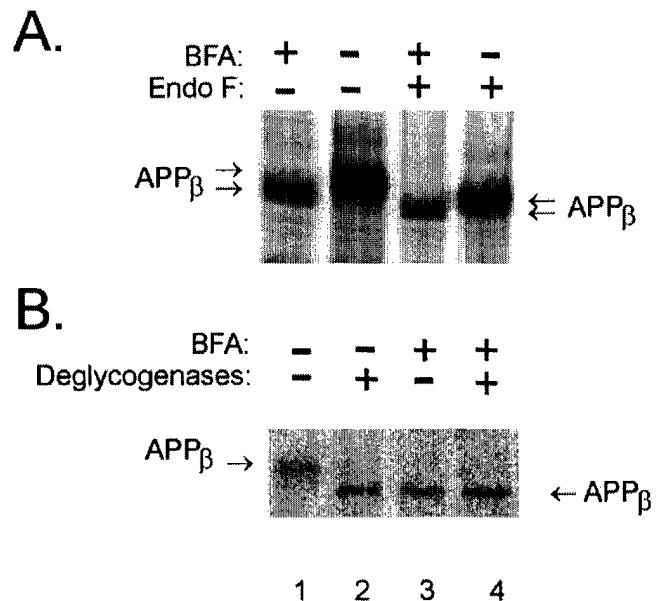


Figure 8. APP_{β} generated in the presence of BFA is partially glycosylated. Cultures of NT2N neurons were metabolically labeled as in Fig. 7 in the presence or absence of 20 $\mu\text{g/ml}$ BFA. The cell lysates were then immunoprecipitated with the antibody 53. (*A*) Samples in lanes 3 and 4 were treated with Nglyc F for 16 h to remove N-linked sugars, whereas immunoprecipitates in lanes 1 and 2 were treated with the vehicle. (*B*) Samples in lanes 2 and 4 were deglycosylated with a combination of Nglyc F, neuraminidase, and *O*-glycosidase for 16 h to remove both N- and O-linked chains (lanes 2 and 4); lanes 1 and 3 represent samples that were mock digested.

from BFA-treated NT2N neurons (Fig. 8 *a*, lane 1) migrated more quickly than APP_{β} recovered from untreated cells (Fig. 8 *a*, lane 2). After digestion with Nglyc F, APP_{β} demonstrated a mobility downshift in SDS-PAGE (Fig. 8 *a*, compare lanes 2 and 4). However, APP_{β} from BFA-treated cells (Fig. 8 *a*, lane 3) still migrated faster than APP_{β} from nontreated cells (Fig. 8 *a*, lane 4) despite enzymatic removal of all N-linked carbohydrate chains. Thus, the increased electrophoretic mobility of APP_{β} in the presence of BFA cannot be accounted for solely by differences in N-linked carbohydrate processing.

In addition to N-linked glycosylation, however, APP undergoes a variety of posttranslational modifications, including the addition of O-linked carbohydrate chains. Therefore, we removed both N- and O-linked carbohydrate chains from immunoprecipitated APP_{β} by simultaneous digestion with Nglyc F, *O*-glycosidase, and neuraminidase. As shown, fully deglycosylated APP_{β} (Fig. 8 *b*, lane 2) comigrated with APP_{β} recovered from BFA-treated NT2N neurons (Fig. 8 *b*, lane 3). Furthermore, combined BFA inhibition and deglycosylation (Fig. 8 *b*, lane 4) did not induce a greater mobility shift than either of these treatments alone (Fig. 8 *b*, lanes 2 and 3). Taken together, these results suggest that APP_{β} generated from BFA-treated NT2N neurons may represent β -secretase processing of immature (nonglycosylated) APP_{FL} in a pre-Golgi compartment.

To further verify that β -secretase cleavage indeed occurs early in the biosynthetic pathway of NT2N neurons,

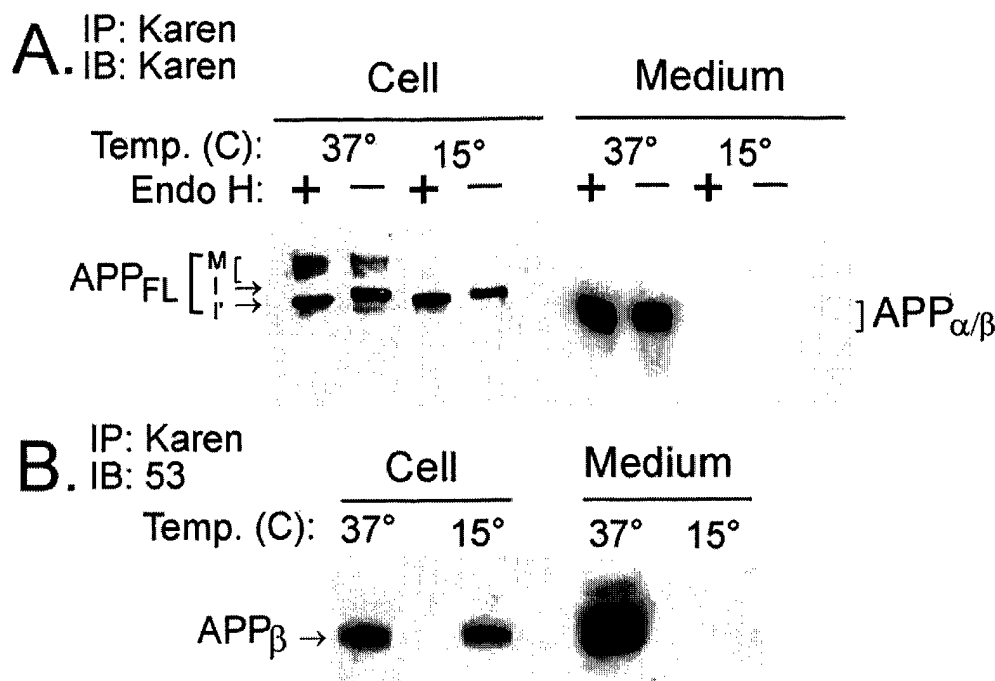


Figure 9. APP β is generated in the ER/IC of NT2N neurons. Approximately 6×10^6 NT2N neurons were incubated at either 15° or 37°C for 16 h. The cell lysates and media were harvested and immunoprecipitated with Karen. (A) The immunoprecipitates were then split, and half of the samples was treated with Endo H for 18 h, while the other half was mock digested. Subsequent to this step, the immunoprecipitates were separated by SDS-PAGE, transferred onto nitrocellulose replicas, and probed with the antibody Karen. The following observations serve to verify the effectiveness of the temperature block: (a) immature forms of APP_{FL} (I and I') in the cell lysate retain Endo H sensitivity at 15°C; (b) mature glycosylated forms of

APP_{FL} (M) in the cell lysate are not detected at 15°C; and (c) secreted fragments are not detected in the conditioned medium at 15°C. (B) Immunoprecipitates were separated by SDS-PAGE, transferred onto nitrocellulose replicas, and probed with antibody 53. APP β continued to be produced intracellularly despite the effective temperature block. However, secreted APP β was not detected in the medium at 15°C. Note that splitting intracellular APP β samples recovered at 15°C for Endo H digestion decreased the yield to below the level of detection by this assay (data not shown). M, mature APP_{FL}; I, immature APP_{FL}; I', immature APP_{FL} demonstrating a mobility shift due to Endo H sensitivity.

we employed an alternative nonpharmacological method to block protein transport from the ER to the Golgi. Incubation of cultured cells at 15°C has been shown to inhibit newly synthesized proteins from exiting the intermediate compartment (Saraste and Kuismanen, 1984; Saraste et al., 1986; Schweizer et al., 1990). To this end, NT2N cells were incubated at 15°C for 16 h. Fig. 9 *a* shows that only the immature form of APP_{FL} was present after a 16-h incubation at 15°C, as indicated by its sensitivity to Endo H digestion, suggesting that it is not transported to the Golgi apparatus under these conditions (Fig. 9 *a*, lanes 3 and 4). By contrast, incubation of the NT2N cells at 37°C yielded both immature and fully processed APP_{FL} (Fig. 9 *a*, lanes 1 and 2). As expected, the immature APP_{FL} was Endo H sensitive, while the mature forms of APP_{FL}, having acquired posttranslational modifications after exiting the ER, were Endo H resistant. In addition, secreted forms of APP were not detected in cells maintained at 15°C, further substantiating the effectiveness of the temperature block. Significantly, continuous production of intracellular APP β was observed at 15°C, despite the fact that the secretion of APP ectodomain is completely abolished (Fig. 9 *b*). Taken together, these data support the ER/IC of NT2N neurons as a β -cleavage site.

A third approach was adopted to confirm that β -secretase cleavage indeed occurs in a pre-Golgi compartment of NT2N neurons. To accomplish this, we compared the processing of wild-type APP695 and APP695 bearing an ER-retrieval motif (APP695_{ΔKK}; Jackson et al., 1990, 1993) in

the NT2N cells. We used recombinant Semliki Forest virus (SFV) vectors to express APP695_{ΔKK}, in which the third and fourth amino acids from the COOH terminus of APP are changed to lysines (i.e., APP695_{ΔKK}). Our previous studies have shown that despite high levels of SFV-mediated APP expression, SFV-infected NT2N cells display a high degree of fidelity in processing APP (Wörtkin et al., 1993; Turner et al., 1996; Cook et al., 1997). Furthermore, we have found that cytopathic effects of SFV infection in NT2N cells as measured by LDH release do not develop until >48 h after infection (data not shown). Importantly, all if not a significant majority of APP695_{ΔKK} colocalize with calnexin, the ER marker, by immunofluorescence upon expression in NT2N neurons (Cook et al., 1997).

To determine whether or not APP β can be produced from APP695_{ΔKK}, wild-type APP695 and APP695_{ΔKK} were separately expressed in NT2N neurons by infection with SFV vectors bearing these constructs. After infection, duplicate wells containing wild-type APP695-infected cells were also treated with 20 μ g/ml BFA. The [³⁵S]methionine-labeled cell lysates and the media were then sequentially immunoprecipitated with the antibodies 53 and Karen. Only the immature form of APP_{FL} was detected from cells expressing APP695_{ΔKK} (Fig. 10 *b*, compare lanes 1 and 3). Significantly, intracellular production and secretion of APP β was not affected by genetic targeting of APP to the ER (Fig. 10 *a*, lanes 3 and 6). Furthermore, we found that unlike inhibition with BFA that eliminates transport of all proteins from the ER to the Golgi, specific retrieval of full

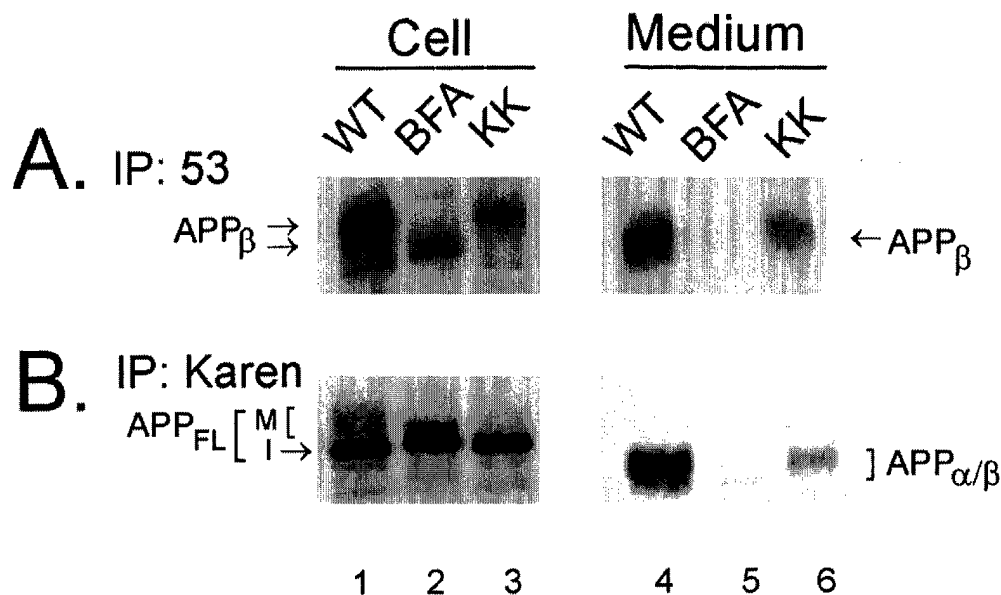


Figure 10. APP β is generated from APP_{FL} that is concentrated in the ER. NT2N cultures of $\sim 1 \times 10^6$ cells were infected with recombinant SFV containing either wild-type APP695 or APP695 Δ KK constructs. The dilysine motif concentrates APP_{FL} to the ER by an efficient retrieval mechanism. Duplicate cultures infected with wild-type APP695 were treated with 20 μ g/ml BFA for comparison. Under these conditions, the cells were metabolically labeled with [35 S]methionine for 16 h. Radiolabeled cell lysates and media were then immunoprecipitated with antibody 53 (for APP β , A) and Karen (for APP_{FL} and APP α/β , B). Radiolabeled immunopre-

cipitates were used to expose PhosphorImager plates (72 h) for visualization of bands. Unlike APP β produced under BFA inhibition, APP β derived from APP695 Δ KK was modified and secreted into the medium.

length APP695 Δ KK to the ER allowed the APP β fragment generated in the ER/IC to be transported to the Golgi complex for modification before secretion (Fig. 10 a, compare lanes 2 and 3 and lanes 5 and 6). This suggests that once the ER retention motif is cleaved from the APP β fragment, it can then be transported to the Golgi complex for further maturation and subsequent secretion.

Discussion

APP serves as a substrate for a variety of proteolytic processing pathways, only some of which result in the production of A β (Selkoe, 1994). However, A β is the major component of senile plaques in the AD brain. Moreover, mutations in the APP gene associated with Familial Alzheimer's disease alter APP processing and A β production in vitro (Citron et al., 1992; Cai et al., 1993; Suzuki et al., 1994). Thus, it will be important to determine the proteolytic events that lead to A β production and to identify the proteases responsible for each step as well as the sites of their action. In addition, it will be important to consider the cell type in which these processes occur. Non-neuronal cells favor the nonamyloidogenic α -secretase pathway. By contrast, neuronal cells exhibit increased β -secretase activity (Busciglio et al., 1993; Wertkin et al., 1993). To better understand APP processing in neurons, we have used the NT2N system for this study. We have previously shown that NT2N neurons express the isoform of APP expressed almost exclusively in the CNS (i.e., APP695) and that they constitutively produce intracellular and secreted A β . In this study, we have identified and characterized some of the intracellular β -secretase activities that cleave on the NH₂ terminus side of A β by using specific antibodies to APP β and to other proteolytic fragments. More significantly, however, we have used three independent approaches

to document novel β - and γ -secretase activities that occur in a pre-Golgi compartment.

Several lines of evidence presented here demonstrate that APP β is derived from APP_{FL} within the cell before secretion. First, APP β was recovered from NT2N cell lysates even after intact NT2N neurons were treated with trypsin. Such treatment would eliminate cell surface-associated APP β but not intracellular APP β . Indeed, the loss of APP β after trypsin treatment of detergent-permeabilized NT2N neurons further confirms the intracellular origin of APP β in NT2N neurons. Second, the continuous presence of steady state levels of APP β in NT2N neurons, together with a delay in the detection of APP β in freshly replenished medium, suggested that APP β is generated intracellularly before secretion. Third, pulse-chase experiments demonstrated that the turnover of intracellular APP β lags behind the turnover of newly synthesized APP_{FL}, thereby confirming that APP β is generated from APP_{FL} inside NT2N neurons before secretion.

The detection of APP β in the cell lysate of NT2N neurons, together with the presence of A β ₄₀ and A β ₄₂ (Turner et al., 1996), firmly established that an intracellular β -secretase pathway(s) must exist in these cells. At present, no other cell line has been reported to produce detectable levels of intracellular APP β from endogenous or over-expressed wild-type APP (Seubert et al., 1993; Haass et al., 1995a; Thinakaran et al., 1996b). Only human kidney 293 cells stably transfected with APPsw cDNA yield the related APP β sw fragment from the cell lysates (Haass et al., 1995a; Martin et al., 1995). In these non-neuronal cells, however, treatment with BFA completely eliminates APP β sw and A β production (Haass et al., 1995a; Martin et al., 1995; Esalmani et al., 1996). In contrast, NT2N neurons continue to produce APP β and A β during treatment with BFA, implying that the subcellular site(s) of the β -secretase pathway is cell-type specific. Furthermore, this lack of inhibi-

tion of APP β and A β production by BFA in NT2N cells suggests that at least one of the β -secretase pathways is localized to the ER/IC. Two additional independent means of testing this hypothesis (i.e., the use of 15°C temperature block and expression of APP bearing the dilysine ER retrieval signal) yielded consistent results.

Our data also suggest that the β -secretase pathway, but not the α -secretase pathway, occurs inside NT2N neurons. This view is based on the absence of APP α and p3 fragments in NT2N cell lysates. Of course, this observation alone cannot rule out the possibility of their presence below the level of detection by our assay. Nevertheless, these results imply that at least in this regard, NT2N neurons are similar to almost all other cell lines in which the enzymes of the α -secretase pathway are active at or near the cell surface. The uniqueness of intracellular processing in postmitotic neuronal cells such as the NT2N neurons lies in the fact that unlike non-neuronal cells, the amyloidogenic β -secretase pathway(s) is preferred. Accordingly, the level of A β secretion is much higher than that of p3 in postmitotic NT2N neurons.

The effect of the Swedish mutation on APP processing is interesting. Overexpression of APP Sw in transfected, non-neuronal cells results in a 5–10-fold increase in A β secretion (Citron et al., 1992; Cai et al., 1993). Concomitant with this change, intracellular APP βSw is also detected in non-neuronal cells stably transfected with APP Sw (Haass et al., 1995a; Thinakaran et al., 1996b). Transfection of wild-type APP695 in non-neuronal cells, however, fails to produce intracellular APP β and results in the secretion of more p3 than A β (Thinakaran et al., 1996b). Thus, it appears that the introduction of the Swedish mutation shifts APP processing away from the α -secretase pathway to the β -secretase pathway. However, unlike NT2N neurons that may use multiple β -secretase pathways to produce both intracellular A β and APP β , APP Sw expressing non-neuronal cells use primarily the endosomal/lysosomal pathway or the Golgi-derived vesicles to generate intracellular A β and APP βSw , since treatment of these cells with BFA completely inhibits APP βSw and A β production (Haass et al., 1995a; Martin et al., 1995).

In view of the foregoing, three potential β -secretase pathways have been identified to date. Of these three, the endosomal/lysosomal pathway, which processes APP targeted to the cell surface after its reinternalization into endosomes and lysosomes, is the most ubiquitous. Both primary cultures of neuronal and non-neuronal cells, as well as multiple cell lines, use this pathway to produce A β . However, the contribution of endosomal/lysosomal processing to the overall production of A β is relatively minor since non-neuronal cells transfected with wild-type APP produce mostly p3 and very little A β (Haass et al., 1992a, b; Koo and Squazzo, 1994; Lai et al., 1995; Thinakaran et al., 1996b). In contrast, an alternative β -secretase pathway that produces A β in Golgi-derived vesicles is the most important for the production of A β in cells transfected with APP Sw . Consistent with this view, transfection of an APP Sw construct lacking the cytoplasmic tail, which eliminates reinternalization of cell surface APP Sw , does not reduce the secretion of A β (Haass et al., 1995a; Essalmani et al., 1996). It is likely that the neuron-like NT2N cells also use this β -secretase pathway since neuronal cells (including

hippocampal neurons and NT2N neurons) produce much higher levels of A β than p3. Finally, the third β -secretase pathway localized to the ER/IC appears to be preferentially used by postmitotic neuronal cells, since intracellular APP β was not detected in several non-neuronal cell lines when treated with BFA.

The possibility of A β generation in the ER of NT2N neurons identifies these cells as a unique system in which to test the hypothesis that amyloidogenic processing of APP within that compartment plays an important role in the pathogenesis of AD. There is now strong evidence that mutations in both the APP gene and the recently identified presenilin genes cause AD by altering APP processing in ways that lead to the production of more amyloidogenic form of A β (i.e., A β_{42} ; Scheuner et al., 1996). Recently, in both non-neuronal and neuronal cells (including the NT2N neurons used in this study), the presenilin proteins have been localized to the ER (Cook et al., 1996; Kovacs et al., 1996; Thinakaran et al., 1996a). Thus, the identification of amyloidogenic processing that may occur within the ER of neurons raises the formal possibility that direct or indirect interaction may occur between the presenilins and APP. Furthermore, the mutations in the presenilin genes may alter this interaction in a manner that leads to increased production of A β_{42} . Therefore, it will be particularly interesting to examine the effects of both Familial Alzheimer's disease-linked mutations occurring in the APP as well as the presenilin genes on the processing of APP in the ER.

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Characterization of the Interaction between the Wilson and Menkes Disease Proteins and the Cytoplasmic Copper Chaperone, HAH1p*

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Wilson disease (WD) and Menkes disease (MNK) are inherited disorders of copper metabolism. The genes that mutate to give rise to these disorders encode highly homologous copper transporting ATPases. We use yeast and mammalian two-hybrid systems, along with an *in vitro* assay to demonstrate a specific, copper-dependent interaction between the six metal-binding domains of the WD and MNK ATPases and the cytoplasmic copper chaperone HAH1. We demonstrate that several metal-binding domains interact independently or in combination with HAH1p, although notably domains five and six of WDp do not. Alteration of either the Met or Thr residue of the HAH1p MTCXXC motif has no observable effect on the copper-dependent interaction, whereas alteration of either of the two Cys residues abolishes the interaction. Mutation of any one of the HAH1p C-terminal Lys residues (Lys⁵⁶, Lys⁵⁷, or Lys⁶⁰) to Gly does not affect the interaction, although deletion of the 15 C-terminal residues abolishes the interaction. We show that apo-HAH1p can bind *in vitro* to copper-loaded WDp, suggesting reversibility of copper transfer from HAH1p to WD/MNKp. The *in vitro* HAH1p/WDp interaction is metalospecific; HAH1p preincubated with Cu²⁺ or Hg²⁺ but not with Zn²⁺, Cd²⁺, Co²⁺, Ni²⁺, Fe³⁺, or Cr³⁺ interacted with WDp. Finally, we model the protein-protein interaction and present a theoretical representation of the HAH1p-Cu-WD/MNKp complex.

Copper is an essential trace element that serves as a cofactor for a number of oxygen-processing enzymes involved in diverse biological processes. For example, cytochrome c oxidase is essential for respiration, dopamine β -hydroxylase is essential for catecholamine formation, superoxide dismutase is essential for free radical detoxification, lysyl oxidase is essential for maturation of connective tissue, ceruloplasmin is essential for iron uptake, peptide- α -amidating enzyme is essential for pituitary peptide hormone maturation, and monophenol monooxygenase is essential for melanin synthesis (1–3). Although the precise mechanisms are unknown, copper plays additional roles in hemoglobin synthesis, angiogenesis, nerve myelination, endorphin action, extracellular matrix stabilization, leukocyte differentiation, and neutrophil and granulocyte maturation (3–6). In

excess, both cupric and cuprous ions are highly toxic, because they act as electron transfer intermediates and catalyze the formation of hydroxyl radicals. This copper-induced production of reactive oxygen species results in DNA damage, as evidenced by strand breaks and by base oxidation of guanosine within cellular DNA and in lipid peroxidation of membranes, especially in mitochondria and lysosomes (7). Thus, proper copper trafficking is essential to cell vitality.

Dietary copper is absorbed into the body through the intestinal mucosa where it joins recycled endogenous copper secreted into the gastrointestinal tract from other tissues. In general, dietary copper absorption is dependent upon the amount of copper reabsorbed from the fluids of other tissues. Newly absorbed copper is transported to body tissues in two phases. First, albumin, transcuprein, amino acids, and a group of uncharacterized low molecular weight proteins transport the majority of exchangeable copper to the liver. After traversing the basolateral membrane of hepatocytes, copper is distributed to endogenous copper-requiring enzymes and to secreted cuproproteins such as ceruloplasmin, which is thereafter released to plasma for delivery of copper to other tissues. Any excess copper is excreted to the bile by the way of the canalicular (apical) plasma membrane (see Refs. 3 and 4 for reviews).

Two P-type ATPases have recently been characterized in humans, ATP7A and ATP7B (8–12). Mutations in these two genes lead to disorders of copper starvation (Menkes disease) and copper toxicity (Wilson disease), respectively. ATP7A is expressed in all tissues except liver, and mutations in this gene prevent the normal absorption and distribution of copper throughout the body. The resulting copper depletion leads to multi-system disorder and death in childhood. The ATP7B gene is expressed in most tissues but predominantly in the liver. Mutations in this gene lead to excessive copper buildup in the liver. A characteristic feature of WD¹ is the abnormally low levels of active (copper-bound) ceruloplasmin. Recent studies show that the molecular components of copper trafficking pathways are highly conserved between yeast and humans. In *Saccharomyces cerevisiae*, the pathway begins with Cu⁺ uptake through the action of copper transporters CTR1p and CTR3p (13, 14). In the cytoplasm, copper is bound by cytoplasmic copper chaperones that then deliver the metal to specific target enzymes (15–21). The ATX1p copper chaperone appears to deliver copper to CCC2p, the yeast homologue of the WD and MNK ATPases (15–17). CCC2p is then required to transfer

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¹ The abbreviations used are: WD, Wilson disease; MNK, Menkes disease; BCA, bathocuproinedisulfonic acid; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; Tricine, N-tris(hydroxymethyl)methylglycine; MBP, maltose-binding protein.

copper from the cytosol to the lumen of the trans-Golgi network where the multi-copper ferroxidase Fet3p, yeast homologue of ceruloplasmin, is loaded (22, 23).

Lin and Culotta (15) provided the first biochemical evidence for interaction between ATX1p and CCC2p. Based on their subcellular localization, Lin and Culotta (15) suggested that ATX1p works as a cytoplasmic copper carrier protein that delivers copper from CTR1p to CCC2p. Recently, Pufahl *et al.* (17) used the yeast two-hybrid protocol to demonstrate a copper-dependent interaction between ATX1p and CCC2p.

In this study we show that human homologues of the yeast proteins ATX1p and CCC2p interact in a copper-dependent manner. Using three independent assays we characterize the interaction of HAH1p (Human ATX1-like Homolog) with the homologous Wilson disease (Wdp) and Menkes disease (MNKp) proteins. The WD and MNK genes encode six MTCXXC motifs in the N-terminal portion of their proteins, whereas their prokaryotic and yeast counterparts typically encode one or two such motifs (24–26). Using deletion constructs of the Wdp metal-binding domain, we provide evidence that a single MTCXXC motif is sufficient to interact with the HAH1p chaperone. Mutagenesis analysis demonstrates that the interaction is dependent on the co-coordination of copper ions by cysteine residues within the MTCXXC motifs of the interacting HAH1 and WD proteins. The data suggest a mechanism for the intermolecular transfer of copper ions similar to the "bucket brigade" model previously proposed for the trafficking of toxic ions in bacterial mercury detoxification (27, 28).

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The entire coding region from a human liver HAH1 cDNA was amplified by polymerase chain reaction and cloned into the expression vectors pACT2 (CLONTECH), pVP16 (CLONTECH), and pProEx-HTb (Life Technologies, Inc.) to create the plasmid constructs pACT-HAH1, pVP-HAH1, and pHTb-HAH1. To create point mutations within the MTCXXC motif, nucleotide changes were introduced directly into the primers for polymerase chain reaction amplification. Plasmids pACT-WM (without Met), pVP-WM, and pHTb-WM carry HAH1 cDNA with the mutation Met¹⁰ → Val. Plasmids pACT-WT (without Thr), pVP-WT, and pHTb-WT carry HAH1 cDNA with the mutation Thr¹¹ → Ala. Plasmids pACT-WC1 (without first Cys), pVP-WC1, and pHTb-WC1 carry an HAH1 cDNA with the Cys¹² → Tyr mutation. Plasmids pACT-WC2 (without second Cys), pVP-WC2, and pHTb-WC2 carry an HAH1 cDNA with the Cys¹⁵ → Tyr mutation. Plasmids pACT-W15N, pVP-W15N, and pHTb-W15N encode HAH1p without the 15 N-terminal amino acid residues. Plasmids pACT-W15C, pVP-W15C, and pHTb-W15C encode HAH1p without the 15 C-terminal amino acid residues. Plasmids pACT-HAH-C12G and pACT-HAH-C15G carry an HAH1 cDNA with a Cys → Gly mutation in the first and second cysteines of the MTCXXC motif, respectively. Plasmids pACT-HAH-K56G, pACT-HAH-K57G, and pACT-HAH-K60G harbor Lys → Gly mutations at one of the Lys residues located at the C-terminal region of HAH1p. A fragment of WD cDNA encoding the entire metal-binding domain of Wdp (coordinates Leu¹⁹–Ile⁵⁹⁹) was cloned into the expression vectors pAS2–1 and pM (CLONTECH) to produce the plasmids pAS-CBM16 (copper-binding motifs 1–6) and pM-CBM16, respectively. The plasmid pMAL-c2/N-WD encodes a fusion of the maltose-binding protein and the metal-binding domain of Wdp and has been described previously (29). Plasmids pAS-CBM15, pAS-CBM14, pM-CBM14, pAS-CBM56, pAS-CBM13, pAS-CBM24, pAS-CBM23, pAS-CBM1, pAS-CBM2, and pAS-CBM3 encode fragments of the metal-binding domain of the WD protein containing metal-binding motifs 1–5, 1–4, 1–4, 5–6, 1–3, 2–4, 2–3, 1, 2, and 3, respectively. Plasmids pAS-CBM14-W1 and pAS-CBM1-WMCC are identical to pAS-CBM14 and pAS-CBM1, respectively, except both harbor an altered first metal-binding motif (MTCQSC → ATAQSA). These alterations were implemented with the Muta-Gene Phagemid *in vitro* mutagenesis kit (BioRad). Plasmid pAS-MNK encodes a GAL4 DNA-binding domain-MNKp metal-binding domain fusion protein. All constructs were verified by sequence analysis. DNA sequencing was performed with the dideoxy chain termination method using the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc.) as recommended by the supplier. The reactions were analyzed on an ABI model 373A automated

sequencer.

Yeast Two-hybrid Assay—The yeast two-hybrid assay was performed according to the protocol supplied by the yeast two-hybrid system supplier (CLONTECH).

Mammalian Two-hybrid Assay—HepG2 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal calf serum, 100 μ M non-essential amino acids, 100 units/ml penicillin, and 100 μ g/ml streptomycin, at 37 °C in 5% CO₂. Cells were seeded at a density of 3.2×10^6 /100-mm tissue culture dish the day before transfection. Transfection of HepG2 cells with plasmid DNA was performed using the DOSPER liposomal transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. 12.5 μ g of both the pM- and pVP16-based plasmids, 2.5 μ g of reporter plasmid pG5CAT, and 100 μ l of DOSPER reagent were used per dish. Four days after transfection the cells were lysed, and CAT expression was assayed using the CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals).

In Vitro Interaction Assay—*Escherichia coli* SG20050 cells were transformed with the plasmids pHTb-HAH1, pHTb-WM, pHTb-WT, pHTb-WC1, pHTb-WC2, pHTb-W15N, and pHTb-W15C. Proteins were expressed using the ProEx-HT Prokaryotic Expression system (Life Technologies, Inc.) and purified using the nickel nitrilotriacetic acid spin kit (Qiagen). The amylose resin bound fusion product, maltose-binding protein-Wdp metal-binding domain, was purified essentially as described by Lutsenko *et al.* (29). Maltose-binding protein was obtained from New England Biolabs. Protein concentration was measured by the Bradford method using a Bio-Rad protein assay kit with bovine serum albumin as a standard. The histidine tag was removed by cleavage with TEV protease (Life Technologies, Inc.) followed by chromatography on nickel nitrilotriacetic acid spin columns and dialysis. Purified proteins were loaded with copper by incubation in a solution containing 15 μ M cupric chloride in TBS-DTT (Tris-buffered saline with 25 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, pH 7.5) at 25 °C for 4 h. Excess copper was removed by overnight dialysis in TBS-DTT at 4 °C. 200 μ g of HAH1p (normal or mutated, loaded or not loaded with copper) was incubated with 1 ml of TBS-DTT containing either 200 μ g of amylose resin-bound fusion product of maltose-binding protein-metal-binding domain of the Wdp or 100 μ g of amylose resin-bound maltose-binding protein at 25 °C for 3 h with shaking. The resin was washed four times with TBS-DTT, and proteins were eluted with 10 mM maltose in TBS-DTT. Equal aliquots of eluted proteins were analyzed by SDS-PAGE in a Tris-Tricine buffer system. The gel system consisted of a 16.5% separating gel, a 10% spacer gel, and a 4% stacking gel, each made with a 32:1 ratio of acrylamide:bisacrylamide. After gel separation, proteins were stained with Coomassie Brilliant Blue G-250.

RESULTS

Yeast Two-hybrid Analysis of the Wdp Metal-binding Domain and HAH1p—To test whether HAH1p transfers copper directly to Wdp, we used the yeast two-hybrid assay. The entire 68-amino acid coding region of HAH1 cDNA was fused in frame to the activation domain of GAL4 (in pACT2 vector), whereas a fragment of WD cDNA encoding 623 N-terminal amino acids was fused in frame to the GAL4 DNA-binding domain (in pAS2–1 vector). The WD protein is predicted to possess eight membrane-spanning domains along with four cytoplasmic domains. The six MTCXXC metal-binding motifs are all encoded within the 623-amino acid N-terminal cytoplasmic domain. The two-hybrid plasmids were co-introduced into *S. cerevisiae* Y187 cells, which contain both the *lacZ* and *his3* reporter genes with upstream GAL4-binding sites. Interaction of the two fusion proteins is necessary to juxtapose the GAL4 DNA-binding and activation domains, which then activate transcription of the reporter genes. Results of these experiments are shown in Fig. 1. The experiment was conducted with vector plasmids only (lane 1), single vector plasmid plus single fusion construct (lanes 2 and 3), both fusion constructs (lane 4), and laminin fusion construct plus HAH1p fusion construct (lane 5). It is clear that the β -galactosidase reporter gene is only activated in yeast cells expressing both the Wdp metal-binding domain and HAH1p fusion proteins (Fig. 1, lane 4). These results indicate that HAH1p interacts directly with the

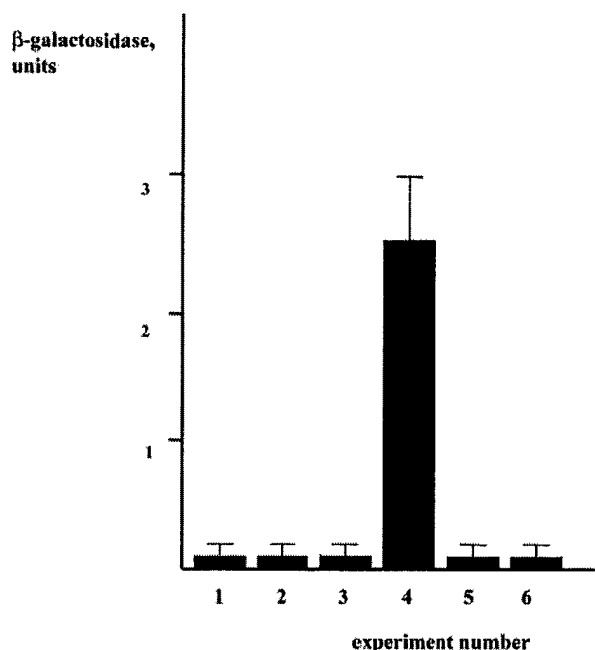


FIG. 1. Yeast two-hybrid assay of the Wdp metal-binding domain and HAH1p. Y187 cells were cotransformed with pAS2-1 and pACT based plasmids, and β -galactosidase activity was measured using *o*-nitrophenyl- β -D-galactopyranoside as substrate. β -Galactosidase activity is shown as the average of five independent transformants \pm S.D. Cells were transformed with the following plasmids (see under "Plasmid Constructs"): lane 1, pAS2-1 (GAL4 DNA-binding domain alone) and pACT2 (GAL4 activation domain alone); lane 2, pAS2-1 and pACT-HAH1 (hybrid of the GAL4 activation domain and HAH1); lane 3, pAS-CBM16 (hybrid of the GAL4 DNA-binding domain and Wdp metal-binding domains 1-6) and pACT2; lane 4, pAS-CBM16 and pACT-HAH1; lane 5, pLAM5' (hybrid of the GAL4 DNA-binding domain and the unrelated protein, laminin) and pACT-HAH1; lane 6 is the same as lane 4 except that cells were grown in the presence of 3 mM BCA, a copper chelator.

N-terminal domain of Wdp. The strength of signal (2.72 β -galactosidase units) was low relative to positive control plasmids (176.5 β -galactosidase units; data not shown) but much higher than negative controls (0.01–0.04 β -galactosidase units; Fig. 1, lanes 1–3 and 5). We observed the same strength of interaction when the two proteins were cloned in opposite vectors (data not shown). All results obtained from the β -galactosidase assay using *o*-nitrophenyl- β -D-galactopyranoside as substrate were confirmed both by substituting 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside as substrate and in yeast mating experiments that measure activation of the alternative reporter gene, *his3* (data not shown). The presence of 3 mM BCA in the growth medium abolishes the interaction of Wdp and HAH1p (Fig. 1, lane 6), suggesting that copper is required for this interaction.

Characterization of the Six Wdp Metal-binding Domains for the Ability to Interact with HAH1p—In the next set of experiments, we sought to determine which MTCXXC motifs, or combination of motifs, are required for interaction with HAH1p. Results of the yeast two-hybrid analysis are shown in Fig. 2. Fusion constructs were generated that contain the full complement of six metal-binding motifs (lane 1) along with various combinations of motifs beginning with the N-terminal most motif 1 to the C-terminal most motif 6: 1–5 (lane 2), 1–4 (lane 3), 5 and 6 (lane 4), 1–3 (lane 5), 2–4 (lane 6), 2 and 3 (lane 7), 1 only (lane 8), 2 only (lane 9), and 3 only (lane 10). The various combinations and numbers of WD metal-binding motifs have only marginal effect on ability to interact with HAH1p with two exceptions. The four N-terminal most motifs (lane 3) promote approximately five times more activity than full length peptide (lane 1), whereas the two C-terminal most mo-

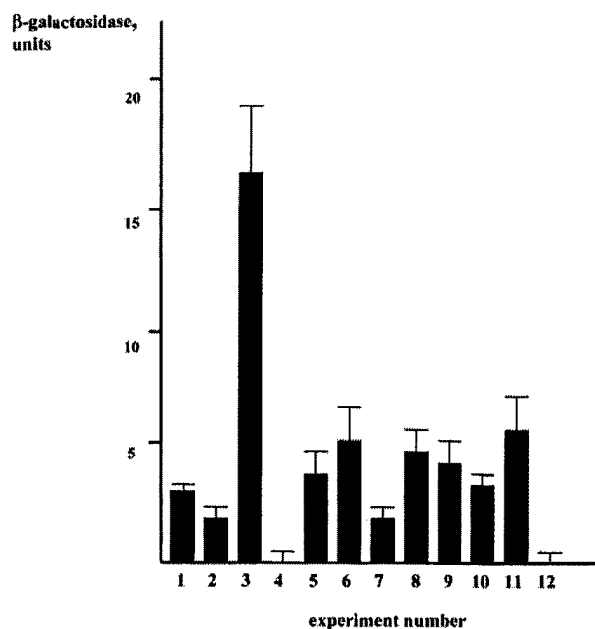


FIG. 2. Characterization of HAH1p/WDp interaction with protein fragments harboring various combinations of the six Wdp metal-binding domains. Y187 cells were cotransformed with pACT-HAH1 (hybrid of the GAL4 activation domain and HAH1) and one of the following plasmids, and then β -galactosidase activity was measured. Lane 1, pAS-CBM16 (hybrid of the GAL4 DNA-binding domain and Wdp metal-binding domains 1–6); lane 2, pAS-CBM15 (domains 1–5); lane 3, pAS-CBM14 (domains 1–4); lane 4, pAS-CBM56 (domains 5–6); lane 5, pAS-CBM13 (domains 1–3); lane 6, pAS-CBM24 (domains 2–4); lane 7, pAS-CBM23 (domains 2–3); lane 8, pAS-CBM1 (domain 1); lane 9, pAS-CBM2 (domain 2); lane 10, pAS-CBM3 (domain 3); lane 11, pAS-CBM14-W1 (domains 1–4 plus the MTCQSC \rightarrow ATAQSA alteration in the first metal-binding site); lane 12, pAS-CBM1-WMCC (domain 1 plus the MTCQSC \rightarrow ATAQSA alteration in the metal-binding site).

tifs generate no evidence of interaction (lane 4). With these two exceptions, various combinations of one, two, or three metal-binding motifs promote virtually the same amounts of β -galactosidase activity. Alteration of the MTCXXC motif abolishes the interaction as shown by comparison of intact motif 1 (lane 9) and motif 1 containing the MTCQSC \rightarrow ATAQSA alteration (lane 12).

Characterization of the HAH1p MTCXXC Motif—In the next set of experiments, we used direct mutagenesis to systematically alter each of the four conserved amino residues in the single MTCXXC motif of the HAH1 protein and then assayed the mutant proteins for copper mediated interaction with Wdp. Fig. 3 shows that normal HAH1p (lane 2) and HAH1p with amino acid alterations at MTCXXC (lane 4) and MTCXXC (lane 5) interact with the metal-binding domain of Wdp. By contrast, alterations at MTCXXC and MTCXXC residues (lanes 6, 7, 12, and 13) abolish the interaction. We also see in Fig. 3 that omission of the N-terminal (lane 3) or C-terminal (lane 8) 15 amino acid residues of HAH1p likewise disrupts the interaction. In addition to the MTCXXC motif at the N terminus of HAH1p, this protein also harbors three lysine residues at its C terminus, which are highly conserved among eukaryotes. In Fig. 3 we show that mutations Lys⁵⁶ \rightarrow Gly, Lys⁵⁷ \rightarrow Gly, and Lys⁶⁰ \rightarrow Gly do not affect the interaction between HAH1p and Wdp as measured by the yeast two-hybrid system (lanes 9–11).

Assay of HAH1p-Cu-Wdp Interaction in Transformed Liver Cells—To better approximate the *in vivo* environment of a putative copper trafficking interaction between the HAH1 and WD proteins, we next measured the interaction in HepG2 hepatoma cells. Fragments of the WD cDNA encoding either

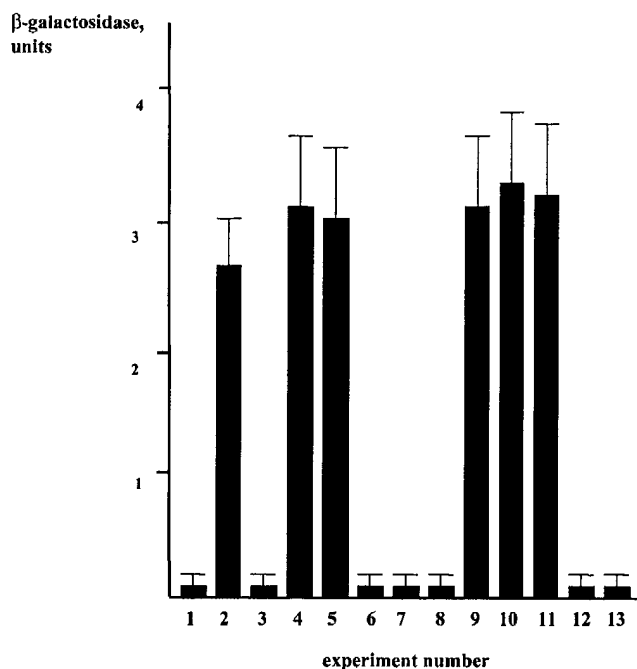


FIG. 3. Yeast two-hybrid assay of the Wdp metal-binding domain interaction with HAH1p constructs containing mutations in the MTCXXC motif. Y187 cells were cotransformed with pAS-CBM16 (hybrid of the GAL4 DNA-binding domain and Wdp metal-binding domains 1–6) and one of the following plasmids, after which, β -galactosidase activity was measured. Lane 1, pACT2 (GAL4 activation domain alone); lane 2, pACT-HAH1 (hybrid of the GAL4 activation domain and HAH1); lane 3, pACT-HAH-W15N (hybrid of the GAL4 activation domain and HAH1; 15 N-terminal amino acid residues of HAH1 are deleted); lane 4, pACT-HAH-WM (contains the Met¹⁰ → Val mutation in HAH1p); lane 5, pACT-HAH-WT (Thr¹⁰ → Ala mutation in HAH1p); lane 6, pACT-HAH-WC1 (Cys¹² → Tyr); lane 7, pACT-HAH-WC2 (Cys¹⁵ → Tyr); lane 8, pACT-HAH-W15C (15 C-terminal amino acid residues of HAH1 are deleted); lane 9, pACT-HAH-K56G (Lys⁵⁶ → Gly); lane 10, pACT-HAH-K57G (Lys⁵⁷ → Gly); lane 11, pACT-HAH-K60G (Lys⁶⁰ → Gly); lane 12, pACT-HAH-C12G (Cys¹² → Gly); lane 13, pACT-HAH-C15G (Cys¹⁵ → Gly).

623 or 499 N-terminal amino acid residues were cloned into the pM vector to produce fusion products of six and four Wdp metal-binding domains, respectively, with the GAL4 DNA-binding domain. The entire coding region of the HAH1 gene was cloned into the pVP16 vector to produce a fusion product consisting of the activation domain of the herpes simplex virus VP16 protein and HAH1p. We co-transfected HepG2 cells with pM and pVP16-based plasmids plus a reporter plasmid (pCAT) containing a CAT gene downstream of four GAL4-binding sites and minimum adenovirus E1b promoter. Interestingly, fusion proteins containing all six metal-binding motifs interact minimally, if at all, with HAH1p (Fig. 4, lane 2), whereas protein encoding the four N-terminal most motifs interact more strongly (lanes 3–5). Interaction between Wdp and HAH1p is blocked by altering either cysteine group (lanes 6 and 7) or by deleting either the 15 N-terminal most (lane 8) or 15 C-terminal most (lane 9) amino acid residues of HAH1p. Alteration of the methionine or threonine residue of the MTCXXC motif of HAH1p does not disrupt the interaction (lanes 4 and 5).

In Vitro Assay of the Interaction between the Wdp Metal-binding Domain and HAH1p—Expression constructs were generated that encode first the entire HAH1 coding segment and second a fusion product consisting of the Wdp metal-binding domain and the maltose-binding protein (MBP). *E. coli* SG20050 cells were transformed with these constructs, and the protein products were purified. HAH1p was subsequently incubated with 15 μ M CuCl₂ in TBS-DTT, followed by removal of unbound copper by dialysis. The MBP-Wdp fusion products

(Fig. 5A, lanes 4 and 5) or MBP alone (lanes 2 and 3) were bound to amylose resin and incubated with preparations of HAH1p that were either loaded (lanes 3 and 5) or not loaded (lanes 2 and 4) with copper. Bound protein was then eluted with 10 mM maltose and analyzed by SDS-PAGE. Fig. 5A shows that the metal-binding domain of the Wdp protein binds specifically to HAH1 protein preloaded with copper (lane 5).

In the next experiment (Fig. 5B) the various mutant forms of HAH1p were preincubated with copper and then incubated with amylose resin-bound MBP-Wdp fusion protein. Proteins bound to the resin were subsequently eluted with maltose and analyzed by SDS-PAGE. Consistent with the yeast and mammalian two-hybrid assays results, intact HAH1p (lane 1), MTCXXC modified HAH1p (lane 3), and MTCXXC modified HAH1p (lane 4) were capable of binding the Wdp fusion protein, whereas MTCXXC (lane 5) and MTCXXC (lane 6) modified HAH1p and HAH1p missing either the N-terminal (lane 2) or C-terminal amino acids (lane 7) were incapable of binding fusion protein. In this experiment, HAH1p constructs were loaded with copper, whereas resin-bound MBP-Wdp fusion protein was copper deficient (see "Experimental Procedures"). The results of the mutagenesis experiments suggest that both Cys residues of the HAH1p MTCXXC motif (copper donor) are required for interaction with Wdp (copper acceptor). The inverse experiment with copper-deficient HAH1p mutants and copper loaded resin-bound MBP-Wdp fusion generated identical results (Fig. 5C), suggesting that both Cys residues of the HAH1p MTCXXC motif (in this case, copper acceptor) are required for interaction with Wdp (copper donor). Thus, four Cys residues, two from the copper donor and two from the copper acceptor, are required for the copper-dependent interaction.

Yeast Two-hybrid Analysis of the HAH1p/MNKp Interaction—The MNK and Wdp proteins are highly homologous, sharing 55% amino acid identity (30). Both proteins contain six metal-binding MTCXXC motifs in their N-terminal portion, as well as a signature inter-membrane CPC motif and all characteristic motifs of P-type ATPases. In this set of experiments, we tested whether MNKp interacts with the HAH1 protein using the yeast two-hybrid assay. Fig. 6 (lane 2) shows that the strength of the HAH1p/MNKp interaction is similar to that of the HAH1p/Wdp interaction (Fig. 1, lane 4). Addition of 3 mM BCA to the growth medium abolishes the protein-protein interaction (Fig. 6, lane 3), suggesting that copper is required for this interaction. The pattern of MNKp interaction with altered variants of HAH1p (Fig. 6, lanes 4–14) is the same as for the Wdp protein (Fig. 3). Amino acid alterations at MTCXXC (Fig. 6, lane 5), MTCXXC (lane 6), Lys⁵⁶, Lys⁵⁷, and Lys⁶⁰ (lanes 10–12) of HAH1p do not affect the HAH1p/MNKp interaction. By contrast, alterations at MTCXXC and MTCXXC (lanes 7, 8, 13, and 14), as well as omission of the N-terminal (lane 4) or C-terminal (lane 9) 15 amino acid residues of HAH1p completely disrupts the interaction.

Metal Specificity of the HAH1p/Wdp Interaction—In these experiments purified HAH1p was preincubated with a variety of metal ions. To test metal specificity of the *in vitro* interaction of the Wdp metal-binding domain and HAH1p, we preincubated HAH1p with different metal ions. After removal of unbound metal by dialysis, HAH1p was incubated with amylose resin-bound MBP-Wdp fusion protein. Proteins bound to the resin were eluted with maltose and analyzed by SDS-PAGE. Fig. 7 shows that the metal-binding domain of Wdp binds to HAH1 protein that has been preincubated with either Cu²⁺ or Hg²⁺ but not with Zn²⁺, Cd²⁺, Co²⁺, Ni²⁺, Fe³⁺, or Cr³⁺.

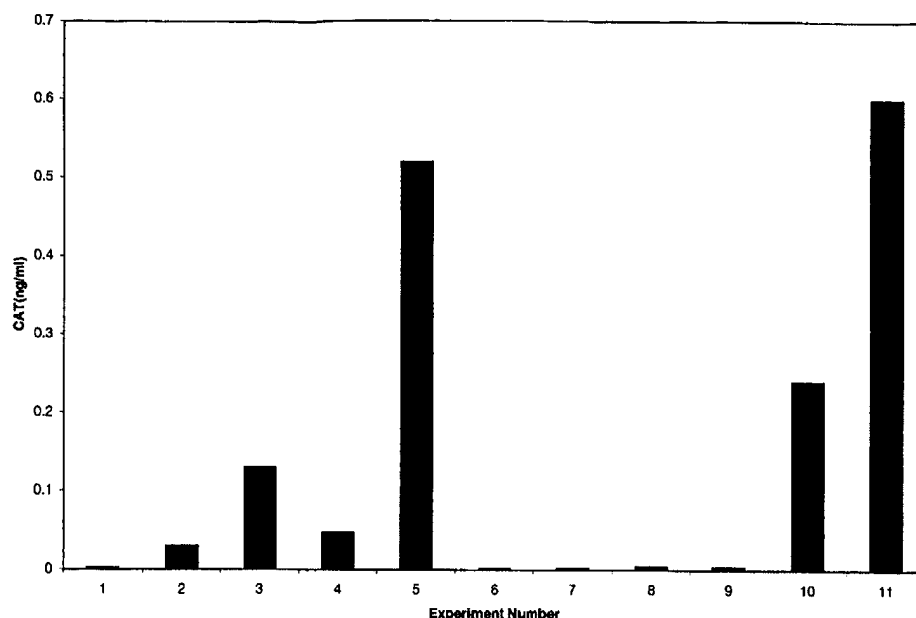


FIG. 4. Measurement of WDP/HAH1p interaction in a mammalian two-hybrid assay. HepG2 cells were cotransfected with three plasmids, pM-X (fusion of the GAL4 DNA-binding domain and the WDP metal-binding domains; see below), pVP16-Y (fusion of the VP16 protein activation domain and HAH1 mutants; see below), and pG5CAT (harbors the CAT reporter gene). Copper was added to the growth medium to a final concentration of 100 μ M. After 4 days the cells were harvested and a CAT enzyme-linked immunosorbent assay was performed using cell lysates. The concentration of CAT detected is proportional to the strength of the interaction between the products of X and Y. CAT concentration is shown as an average for three transfections. Standard deviation does not exceed 10%. Lane 1, untransfected control; lane 2, X = CBM16 (WDP metal-binding domains 1–6), Y = HAH1 (unaltered HAH1); lane 3, X = CBM14 (WDP metal-binding domains 1–4), Y = HAH1; lane 4, X = CBM14, Y = HAH-WM (contains Met¹⁰ \rightarrow Val mutation in HAH1); lane 5, X = CBM14, Y = HAH-WT (Thr¹⁰ \rightarrow Ala mutation in HAH1); lane 6, X = CBM14, Y = HAH-WC1 (Cys¹² \rightarrow Tyr mutation in HAH1); lane 7, X = CBM14WD, Y = HAH-WC2 (Cys¹⁵ \rightarrow Tyr mutation in HAH1); lane 8, X = CBM14, Y = HAH-W15N (15 N-terminal amino acid residues of the HAH1 are deleted); lane 9, X = CBM14, Y = HAH-W15C (15 C-terminal amino acid residues of the HAH1 are deleted); lane 10 (positive control), cells transfected with pM53 (encodes murine p53) and pVP16-T (encodes SV40 large T-antigen); lane 11 (positive control 2), cells transfected with pM3-VP16 (encodes a fusion of the GAL4 DNA-binding domain and the VP16 protein activation domain).

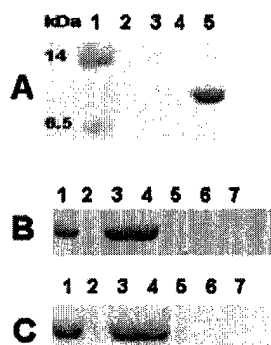


FIG. 5. *In vitro* interaction of the WDP metal-binding domain and HAH1p. A, HAH1p loaded (lanes 3 and 5) or not loaded (lanes 2 and 4) with copper was incubated with amylose resin-bound fusion product maltose-binding protein-metal-binding domain of WDP (lanes 4 and 5) or amylose resin-bound maltose-binding protein only (lanes 2 and 3) as described under "Experimental Procedures." Proteins bound to the resin were eluted with 10 mM maltose and analyzed by SDS-PAGE as described under "Experimental Procedures." Lane 1 shows molecular mass markers. B and C, HAH1p loaded with copper was incubated with amylose resin-bound fusion product maltose-binding protein-WDP metal-binding domain (B) or copper loaded amylose resin-bound fusion product maltose-binding protein-WDP metal-binding domain was incubated with HAH1p (C). Lane 1, unaltered HAH1p; lane 2, HAH1p lacking the 15 N-terminal amino acid residues; lane 3, HAH1p with an alteration Met¹⁰ \rightarrow Val; lane 4, Thr¹¹ \rightarrow Ala; lane 5, Cys¹² \rightarrow Tyr; lane 6, Cys¹⁵ \rightarrow Tyr; lane 7, HAH1p lacking the 15 C-terminal amino acid residues.

DISCUSSION

The Wilson disease gene (ATP7B; WD) encodes a membrane-associated metal transporting P-type ATPase with six metal-binding sites (MTCXXC) located at the N-terminal portion of the protein. The WD gene shares 55% amino acid

identity with the Menkes disease gene (ATP7A; MNK), and all major structural and functional motifs are conserved. Both WD and MNK ATPases have been localized to the trans-Golgi network (23, 31–33) where the proteins are presumably engaged in the transfer of cytoplasmic copper to copper-requiring proteins.

This study addresses the mechanism of copper trafficking whereby cytoplasmic copper is delivered to the WD and MNK ATPases. The elaboration of copper trafficking in humans has followed from experiments with highly homologous proteins in the yeast, *S. cerevisiae*. Recent studies in yeast have shown that copper is taken up by cells through the action of CTR1p and then transferred either directly or indirectly to the cytoplasmic chaperone ATX1p. ATX1p is thought to transfer bound copper to CCC2p, which in turn conveys copper to Fet3p. In this study we show that the human homologue of ATX1p, HAH1p, interacts directly with WDP and MNKp using both yeast and mammalian cell assays and *in vitro* analysis. We further show that two cysteine residues of the HAH1p MTCXXC motif are necessary for the interaction, that a single MTCXXC containing domain of the WD ATPase is capable of interaction with HAH1p, and that copper ion is required for the interaction. We also show that Cu²⁺ and Hg²⁺ but not Zn²⁺, Cd²⁺, Co²⁺, Ni²⁺, Fe³⁺, or Cr³⁺ can mediate the interaction between the Wilson disease and HAH1 proteins.

Our experiments directly assess the contribution of individual MTCXXC motifs in the interaction between the WD protein and HAH1p. The main conclusion from these studies is that several of the individual WD copper-binding motifs are capable of independent interaction with the HAH1 protein. Metal-binding domains 1, 2, and 3 are each sufficient to interact with HAH1p, and various combinations of domains 1–4 appear to interact with roughly equal efficiency. Interestingly, the last

β -galactosidase,
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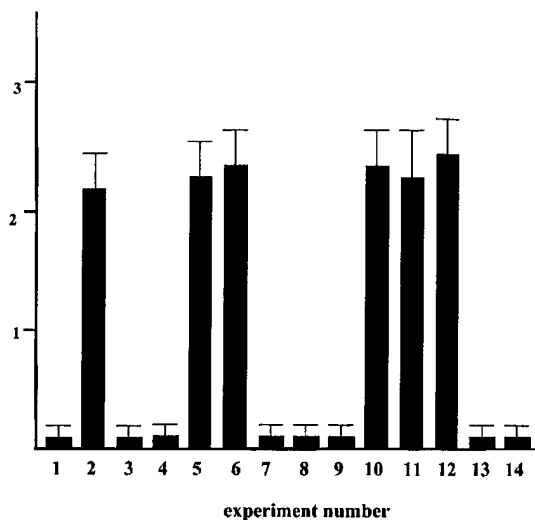


FIG. 6. Yeast two-hybrid assay of the MNKp metal-binding domain with HAH1p. Y187 cells were cotransformed with pAS-MNK (hybrid of the GAL4 DNA-binding domain and MNKp metal-binding domains 2–6) and one of the following plasmids, and then β -galactosidase activity was measured. Lane 1, pACT2 (GAL4 activation domain alone); lane 2, pACT-HAH1 (hybrid of the GAL4 activation domain and HAH1); lane 3, pACT-HAH1 (cells were grown in the presence of 3 mM BCA); lane 4, pACT-HAH-W15N W15N (hybrid of the GAL4 activation domain and HAH1; 15 N-terminal amino acid residues of the HAH1 are deleted); lane 5, pACT-HAH-WM (Met¹⁰ → Val mutation in HAH1); lane 6, pACT-HAH-WT (Thr¹⁰ → Ala mutation in HAH1); lane 7, pACT-HAH-WC1 (Cys¹² → Tyr); lane 8, pACT-HAH-WC2 (Cys¹⁵ → Tyr); lane 9, pACT-HAH-W15C (15 C-terminal amino acid residues of the HAH1 are deleted); lane 10, pACT-HAH-K56G (Lys⁵⁶ → Gly); lane 11, pACT-HAH-K57G (Lys⁵⁷ → Gly); lane 12, pACT-HAH-K60G (Lys⁶⁰ → Gly); lane 13, pACT-HAH-C12G (Cys¹² → Gly); lane 14, pACT-HAH-C15G (Cys¹⁵ → Gly).



FIG. 7. Metal specificity of the HAH1p/WDp interaction. HAH1p was preincubated with different metal ions, and then its interaction with the amylose resin bound fusion construct maltose-binding protein-metal-binding domain of WDp was studied as described under "Experimental Procedures." Proteins bound to the resin were eluted with maltose and analyzed by SDS-PAGE.

two WD metal-binding domains (5 and 6) fail to interact with HAH1p, and the truncated 1–4 domain fragment interacts more strongly than the intact 1–6 domain fragment. The simplest explanation for these results is: first, that the hybrid protein containing WD metal-binding domains 5 and 6 is folded such that the MTCXXC motifs are not exposed on the globule surface and thus are inaccessible for interaction with HAH1p, and second, that under our experimental conditions, the 5–6 fragment masks MTCXXC motifs from the 1–4 fragment. The results indicate that the fifth and sixth copper-binding motifs do not participate directly in the exchange of copper with HAH1p and thus suggest a different function for these motifs.

The interaction of apo-HAH1p with copper-loaded WDp suggests that copper transfer between these proteins is a reversible process. We think that at equilibrium, the HAH1p-Cu-WDp complex can dissociate with an equal chance of forming either HAH1p-Cu + WDp or HAH1p + Cu-WDp. It follows that removal of the Cu-WDp moiety would shift the equilibrium toward the formation of Cu-WDp, whereas removal of HAH1p-Cu would shift the equilibrium toward the transfer of copper from WDp to HAH1p. *In vivo*, removal of the Cu:WDp complex might

be achieved by the transport of copper across the membrane in conjunction with ATP hydrolysis. Thus, our data raise the possibility that HAH1p may function in some circumstances to remove copper from the WD and MNK ATPases, presumably in response to intracellular cues.

In our yeast two-hybrid experiments, HAH1p mutations Lys⁵⁶ → Gly, Lys⁵⁷ → Gly, and Lys⁶⁰ → Gly did not affect interaction between the copper chaperone and either WDp or MNKp. Recent yeast two-hybrid analyses of the yeast HAH1 homologue, ATX1, showed that mutation of the corresponding residues, Lys⁶⁵ → Glu and Lys⁶¹ → Glu + Lys⁶² → Glu, as well as other compound lysine to glutamate mutations, abolish interaction between the copper chaperone and CCC2p, the WD/MNK homologue (35). As the x-ray crystal structures for ATX1p (36) and the fourth metal-binding domain of MNKp (MNK4) have recently been reported (37), we attempted to reconcile these differences by modeling the WDp/MNKp-HAH1p interaction. As shown in Fig. 8, our model indicates that the HAH1p/MNK4 interaction is stabilized by two salt bridges formed between the positively charged HAH1p lysine residues Lys⁵⁷ and Lys²⁵, and the negatively charged MNK4 residues Glu²² and Asp⁶³, respectively. According to this model, a Lys⁵⁷ → Gly mutation would remove one of the stabilizing salt bridges, whereas a Lys⁵⁷ → Glu mutation would both disrupt the salt bridge and introduce a repulsive force that disrupts the protein-protein interaction. The Lys⁵⁶ residue is nearly completely exposed to solvent and thus is unlikely to affect protein-protein interaction. Lys⁶⁰ is close to the copper-binding site as predicted from the ATX1p x-ray structure (36), where it may provide an electrostatic potential gradient that favors the movement of the positively charged copper ion from HAH1p to MNK4. The Lys⁶⁰ → Glu mutation would reverse the gradient and thus trap the copper at the HAH1p copper-binding motif, whereas a Lys⁶⁰ → Gly mutation would predictably have less effect.

Proteins containing the MTCXXC motif are present in such evolutionary distant organisms as bacteria and man. The motif consisting of two cysteine residues separated by any two amino acid residues is absolutely conserved among these proteins from diverse phylogenetic origins. By contrast, the threonine residue is often substituted, and less frequently, MTCXXC containing proteins (for example the CopP protein of *Helicobacter pylori*) lack a methionine residue at this site. A glycine residue often precedes the MTCXXC motif. Functionally characterized MTCXXC proteins are involved in the transport of either copper or mercury, where the cysteine residues are directly involved in the binding of the metal ions. In all three of our test systems mutation of either Met or Thr did not affect interaction between HAH1p and the metal-binding domain of the WD protein, whereas alteration of either Cys residues abolished the interaction. Interestingly, in the work of Hung *et al.* (34), mutations in both Cys residues of the MTCXXC motif of ATX1p were required to eliminate copper incorporation into Fet3p. It is possible that the Met residue can provide a second sulfur atom for bi-coordination of copper in ATX1 proteins containing a single Cys in the metal-binding motif.

It is noteworthy that the copper trafficking mechanism first proposed by Lin and Culotta (15) and Pufal *et al.* (17) from yeast studies and supported by this study of human proteins is closely analogous to the mechanism proposed for mercury detoxification in bacteria (27, 28). In bacteria, the periplasmic protein, MerP, binds Hg²⁺ with its MTCXXC motif and then relays it to the integral membrane proteins MerT and/or MerC (both contain a pair of cysteine residues believed to be involved in Hg²⁺ binding) whose function is to convey Hg²⁺ to the

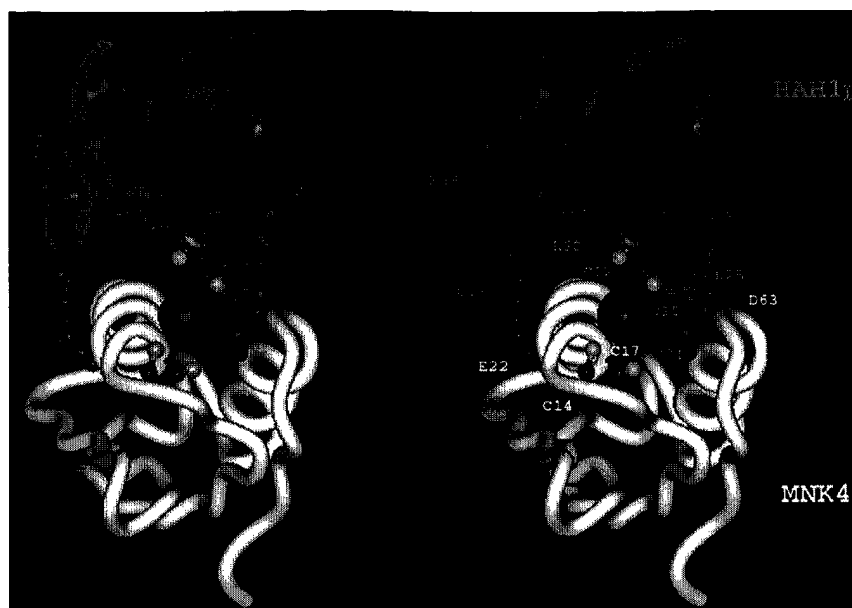


FIG. 8. **Model of the HAH1p-MNK4 complex.** This figure shows a side-by-side stereo view of the theoretical HAH1p-MNK4 complex. The MNK4 x-ray crystal has been reported previously (37) and is illustrated in *white*. Because HAH1p is homologous to MNK4, we adapted the MNK4 structure using the PrISM (protein informatics system for modeling) (38) program to model HAH1p as shown in *blue*. The complex model was built manually, guided by the common four-helix bundle type folding topology at the interface of the HAH1p and the WD/MNK metal-binding domain and by the distorted tetrahedral complex formed by the copper ion (as shown in magenta) and the four cysteines from the copper-binding motifs of the donor and receptor proteins. Based on this model, HAH1p residue Lys⁶⁷ (K57) is in range to form a salt bridge with the Glu²² (E22) residue of MNK4. Glu²² is highly conserved in all WD/MNK metal-binding motifs (data not shown), consistent with a critical role in formation of the donor-acceptor complex. The model also predicts the formation of a critical salt bridge between residues Arg²¹ (R21) and Lys²⁵ (K25) from HAH1p and Asp⁶³ (D63) from MNK4. Negatively charged residues are likewise highly conserved at the Asp⁶³ location among WD/MNK metal-binding domains (data not shown). *Red* depicts negatively charged side groups; *blue* depicts positively charged groups.

cytoplasmic mercury reductase, MerA. MerA catalyzes the reduction of Hg²⁺ to the volatile and less toxic Hg⁰ ion. There is an obvious analogy between the MerP → MerT → MerA pathway and the HAH1p → Wdp → ceruloplasmin pathway (or ATX1p → CCC2p → Fet3p pathway in *S. cerevisiae*). Because unbound copper is highly toxic, it is reasonable to propose a bucket brigade like mechanism for copper trafficking.

We do not yet know whether other copper chaperons (39) mediate the transfer of cytoplasmic copper to the MNK and WD ATPases. We do believe that the copper chaperon proteins can, to some extent, exchange copper among themselves. The cytoplasmic copper chaperon Cox 17p is believed to traffic copper to the mitochondria for insertion into cytochrome *c* oxidase, the terminal oxidase of the respiratory chain (20). Our yeast two-hybrid experiments indicate that HAH1p interacts with Cox17p and that Cox17p, but not HAH1p, interacts with a Cox2p fragment that is presumably located in the mitochondrial intramembrane space (data not shown). Likewise, it is not known how the MNK and WD ATPases transfer copper from the metal-binding domain across the membrane or what role the multiple metal-binding domains play in this regard. The multiple metal-binding domains might serve as traps for copper ions that effectively creates a high local concentration of copper at the intramembrane metal-binding site. Another possibility is that copper binding induces conformational changes in the metal-binding domain that subsequently renders the intramembrane metal-binding site accessible to the cytoplasmic surface. Functional studies will be required to address these questions.

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11783184 99221738 PMID: 10203471
**Serological discrimination of dogs infected with gastric Helicobacter
 spp. and uninfected dogs.**
 Strauss-Ayali D; Simpson K W; Schein A H; McDonough P L; Jacobson R H;
 Valentine B A; Peacock J
 Department of Clinical Sciences, College of Veterinary Medicine, Cornell
 University, Ithaca, New York 14853, USA.
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Characterization of the humoral immune responses of people to *Helicobacter pylori* infection has facilitated the investigation of the host response to bacterial virulence factors and the development of sensitive and specific diagnostic tests. Dogs are commonly infected with gastric *Helicobacter* spp., but the presence of multiple *Helicobacter* spp. and possible coinfection in individual dogs have complicated serological evaluation. Evaluation of the antigenic homology of *Helicobacter* spp. revealed that the major protein bands of *Helicobacter felis* and *Helicobacter bizzozeronii*, two *Helicobacter* spp. that infect dogs, were very similar to UreA (29 to 31 kDa), UreB (63 to 66 kDa), and HSP (58 to 60 kDa) of *H. pylori*, and sera from infected and uninfected dogs bound in a similar way to each antigen. Immunoblotting and an enzyme-linked immunosorbent assay (ELISA) with *H. felis* ATCC 49179 antigen were performed with 101 serum samples (from 78 infected dogs and 23 uninfected dogs). Samples from uninfected dogs (median = 8) had fewer bands on immunoblotting than samples from infected dogs (median = 16) ($P < 0.05$). Combinations of the presence of any two of the low-molecular-mass bands (19, 25, 30, 32, and 37 kDa) or the high-molecular-mass bands (86 and 94 kDa) were found almost solely in samples from infected dogs ($P < 0.0001$). Kinetic ELISA results were significantly higher for samples from infected dogs (median = 0.0802 optical density unit [OD]/min) than for samples from uninfected dogs (median = 0.01428 OD/min). The combination of ELISA and immunoblotting results gave a specificity of 95.6% and a sensitivity of 79.8%. No correlation between ELISA results, colonization density, degree of inflammation, and presence of lymphoid follicles was observed. The results indicate substantial antigenic homology between *H. felis*, *H. pylori*, and *H. bizzozeronii*. The combination of ELISA and immunoblotting was a highly specific and moderately sensitive indicator of infection. The degree of seropositivity assessed by ELISA was not related to bacterial colonization density, the degree of gastric inflammation, or the presence of lymphoid follicles.

Tags: Animal; Female; Support, Non-U.S. Gov't

Descriptors: **Helicobacter* Infections--immunology--IM; **Helicobacter pylori*--immunology--IM; *Stomach--microbiology--MI; Antigens, Bacterial--analysis--AN; Dogs; Enzyme-Linked Immunosorbent Assay; Gastritis--etiology--ET; *Helicobacter* Infections--diagnosis--DI; Immunoblotting; Immunoglobulin G--blood--BL; Molecular Weight; Serologic Tests

CAS Registry No.: 0 (Antigens, Bacterial); 0 (Immunoglobulin G)

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Isolation of *Helicobacter pylori* from human faeces.

Thomas, J.E.; Gibson, G.R.; Darboe, M.K.; Dale, A.; Weaver, L.T.

The Lancet, v340, n8829, p1194(2)

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Helicobacter pylori is arguably the commonest chronic infection in man. However, its route of transmission is unknown. We have isolated viable *H. pylori* from the faeces of an infected individual from The Gambia. The organism was cultured on selective media after concentration of faecal bacteria by centrifugation in a buffer equilibrated with a microaerophilic gas mixture. Growth characteristics, microscopic appearances, and enzyme activities were the same as those of a typical gastric isolate of *H. pylori*.

Protein preparations derived from the new isolate and the typical strain were antigenically similar, and had very similar electrophoretic profiles (including two major protein bands of 62 and 26 kDa, corresponding to the urease enzyme subunits). With the same technique, organisms with the colony morphology, growth requirements, enzyme activities, and microscopic appearances of *H pylori* were isolated from the faeces of 9 of 23 randomly selected children aged 3-27 months from a Gambian village with a high prevalence of *H pylori* infection in early life. Faecal-oral transmission is probably important in the spread of infection in such communities.

Helicobacter pylori is arguably the commonest chronic bacterial infection in man; in parts of Africa it is almost universal among adults, [1] and in The Gambia close to 50% of infants are infected, the percentage being 90% by age 5 years. [2] *H pylori* colonises and has been isolated from the gastric mucosa. [3] Infective bacteria have also been isolated from dental plaque, [4] which indicates that the infection could be transmitted by oral oral secretions. The suggestion that the organism may be excreted in the stool [5] could account for the relation between a high prevalence of infection and poor domestic amenities. [6] The aim of this study was to isolate viable *H pylori* from the faeces of an infected person.

Stool samples from a 24-year-old Gambian man with an established *H pylori* infection diagnosed by [13] C-urea breath test were processed immediately after collection. [7] The man had not received antibiotics for at least 3 months before the study, had no dyspeptic symptoms, and was healthy. A faecal slurry (25% weight/volume) was prepared by homogenising faeces in sodium phosphate buffer (0.1 mol/l; pH 7.0) that had been autoclaved and equilibrated with a microaerophilic gas mixture (5% [O.sub.2], 10% [CO.sub.2] 85% [N.sub.2]) for 24 h. The slurry was then sieved (250 μ m pore size) and the resulting suspension was centrifuged and washed once at 20 000 g for 30 min. Samples of the resultant bacterial layer, a loopful of which produced an instant violet colour with 200 μ l of urease indicator (30 mmol/l urea with phenol red, acidified to the point of yellow colour change), were plated onto selective media (5% horse blood, Colombia agar base with either Dent or Skirrow's antibiotic supplements) and incubated at 37 [degrees] C in a microaerophilic atmosphere for 1 week. Primary incubation produced a mixed growth of bacteria. On almost all plates examined, tiny colonies that produced an immediate violet colour when a small inoculum was introduced into 200 μ l of urease indicator were detected after 5 days. Bacteria from these colonies had the typical appearance of *H pylori* on gram staining. On two separate occasions colonies were subcultured and a pure growth of bacteria was obtained with 5% blood Colombia agar base with Dent or Skirrow's antibiotic supplements. These colonies were catalase positive. Pure subcultures were harvested from plates into distilled water and washed twice.

Antigens were prepared from these subcultures and from an isolate of *H pylori* obtained from a gastric biopsy specimen and examined with an IgG enzyme-linked immunosorbent assay (ELISA). [8] Serum samples from 40 patients who had undergone endoscopy, representing a range of IgG reactivities against *H pylori*, were used to compare antigen preparations. ELISA revealed a significant association between IgG concentrations obtained with either antigen preparation for all 40 serum samples (correlation, [R.sub.2] = 0.695).

The electrophoretic protein profiles of the crude antigens prepared from the stool isolate were compared with those from a strain of *H pylori* from a gastric biopsy specimen. The supernatant obtained after centrifugation was separated by electrophoresis in a sodium dodecyl sulphate/10% polyacrylamide gel using a discontinuous buffer system. [9]

Both preparations contained major bands at 62 kDa and 26 kDa, correspond to the subunits of *H pylori* urease. [8] Other major bands common to both preparations occurred at 14-2 kDa and 45 kDa, whereas differences in minor protein bands were most apparent at 18 kDa, 25 kDa, and 50 kDa.

The same cultural techniques were used to examine faeces from 23 randomly selected children (aged 3-27 months, mean age 13-8 months) from a village in The Gambia, West Africa, where 90% of children are infected with *H pylori* by the age of 5 years. [2] In 9 of the 23 children tiny colonies of bacteria with strong urease activity were detected within a week after culture under the conditions described above. Bacteria from these colonies had the typical microscopic appearances of *H pylori*. The subjects from whom we have successfully grown *H pylori* normally live in the same

Gambian village. The faecal isolate examined in detail had the same microbiological and antigenic characteristics as isolates obtained from the gastric mucosa, and electrophoretic analysis showed that it obtained the same major proteins as a typical gastric isolate. Differences in minor protein bands between the faecal isolate and the control isolate were within the limits of normal variation.[10]

There are two important implications of this report. First, the isolation of viable *H pylori* from faeces shows that infective organisms are present in the colon of some individuals; and second, the high prevalence of *H pylori* infection in West African children[2] is most probably due to faecal-oral spread of infection.

The intraluminal environment of the gastrointestinal tract of West Africans, by supporting the passage of *H pylori* from stomach to colon, and into the faeces, may account for differences in clinical picture of *H pylori* infection between the developed and developing worlds.[1] Exposure of small intestinal lymphoid tissue to bacterial antigen in early life may modify the immune response, which could explain why the clinical features of the infection in adults in West Africa are milder than those in the developed world.[5]

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COMPOSITIONS, TEST KITS AND METHODS FOR DETECTING HELICOBACTER PYLORI
COMPOSITIONS, KITS D'EXAMEN ET PROCEDES POUR DETECTER HELICOBACTER PYLORI
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LV MK MX NO NZ PL RO SG SI SK TR UA US VN YU ZA ZW
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Fulltext Availability:
Detailed Description
Claims

Detailed Description

... expressed as percent IOD, with HP1 , HP2, HP3 and HP4 from all three different H. **pylori** strains Hp504, Hp08 and Hp02 present in sera from 9 patients diagnosed with a H...

...molecular weights 32 kd; 30 kd; 23 kd; and 15 kd and wherein the 15 kd antigen consists of two different **Helicobacterpylori** derived proteins. These antigens from H. **pylori** have not been used in this combination in other available tests. The antigens were characterized...

...HP4a and HP4b) calculated according to the molecular weight standards are as follows: HP1 with - 32 kd , HP2 with - 30 kd , HP3 with - 23 kd , HP4a with -15 kd and HP4b with -15 kd .

The antigens were identified by determining 20-21 N-terminal amino acids and performing a blast search as follows: HP1 was identified to be the response regulator from H. **pylori** (Tomb et al., 1997), HP2 is the 26 kd antigen from H. **pylori** (O'Toole et al., 1991; Tomb et al., 1997), HP3 is the nonheme iron-containing ferritin from H. **pylori** (Doig P. et al., 1992; Frazier B.A. et al., 1993), HP4a is the thioredoxin from H. **pylori** (Tomb et al., 1997) and HP4b is a histone-like DNA-binding protein from H. **pylori** (Tomb et al., 1997).

SIDS PAGE in connection with the invention refers to SDS-Polyacrylamide ...homology search in the database to identify the protein (table 5).

IDENTIFICATION OF THE H. **PYLORI** ANTIGENS.

After microsequencing 20-21 amino acids of the three antigens of H. **pylori** a blast search in different data bases was performed. All proteins could be identified with 85-100% identity with H. **pylori** proteins.

Protein HP4 was a mixture of two components that both could be identified. Table 5 shows the results of that search.

Anti- MW Sequence % Iden- identified as Accession
gen tity* Data Bank
HP1 @ 32 kd MIDVLMIEDHIELAEF 87.5 % Response regulator from 024973
Hp AE000537/EMBL
HP2 - 30 kd @MLVTKLAPDFKAPAVLG I 100 % 26 kd Antigen from Hp 2507172
P21762/Swiss-Prot
HP3 - 23,5 kd MLSKDLIKLLWEQVW 85 % Nonheme iron-containing 477619
ferritin from hp A49694/PIR
HP4a -15 kd SHYIELTEWFESTIKKGVA 100 % Thioredoxin from Hp 3024719
P56430/Swiss-Prot
4b -15 kd MNKAEFIDLKVEAGKYNSKRE 100 % Histone...

...2313970

protein from Hp AE000595/Genbank
in an 16 - 21 aa overlap
Table 5: H. **pylori** antigens identified by microsequencing
-13

Examples

SDS-PAGE AND IMMUNOBLOT ANALYSIS.

The separation of whole cell lysates of three H. **pylori** strains and one C. jejuni strain on tricine gradient gels was performed to show the...

Claim

1 .Composition comprising at least three **Helicobacter pylori** derived proteins, wherein the proteins are selected from the group of **Helicobacter pylori** derived...

...of said **Helicobacter pylori** derived proteins.

5 Composition according to claim 1 wherein the 23 **kd** antigen is a nonheme iron-containing ferritin from **Helicobacter pylori** , one of the 15 **kd** antigens is a thioredoxin from **Helicobacter pylori** and the other 15 **kd** antigen is a histone-like DNA-binding protein from **Helicobacterpylori** .

6 Composition according to claim 1, wherein the antigen specific to **Helicobacter pylori** of molecular weight 32 **kd** represents a response regulator from **Helicobacterpylori** .

7 Composition according to claim 1 wherein two proteins forming the 15 **kd** antigen are present.

8 Composition according to claim 1 wherein the 30 **kd** , the 23 **kd** and at least one of the 15 **kd** antigens is present.

9 Composition according to claim 1, wherein the antigens are present attached...

...labeled antibody is used for detection.

26 In a method for determination the eradication of **Helicobacter pylori** the improvement consisting in the detection of the presence or absence of antibodies resulting...

...of antigens from **Helicobacter pylori** for detecting the presence or absence of antibodies resulting from **Helicobacter pylori** infection wherein the antigens are selected from the group of **Helicobacter pylori** derived proteins which are characterized by SIDS PAGE to consist of antigens specific to **Helicobacter pylori** of molecular weights 32 **kd** ; 30 **kd** ; 23 **kd** ; and 15 **kd** and wherein the 15 **kd** antigen consists of two different **Helicobacter pylori** derived proteins.
Figure IA: Reactivities of H. pylori positive sera with antigens from Hp504
100...

4/3,KWIC/6 (Item 5 from file: 349)
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00558762

i(HELICOBACTER PYLORI) VACCINE

VACCIN ANTI-i(HELICOBACTER PILORI)

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PT SE
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Detailed Description

Detailed Description

... in der ausseren Membran des Erregers zu finden. So sind in der
ausseren Membran von **Helicobacter pylori** Adhasine von 19,6 kD (Doig et
al. (1 992>> und 20 kD (Evans...

...diedie bakterielle Adhasion an der mukosalen Oberflache verhindern.
Ausserdem verfügt die aussere Membran von **Helicobacter pylori** uber
Porine mit Molekulargewichten von 30 kD (Tufano et al. (1 994>>, 48
kD , 49 kD 50 kD , 67 kD (Exner et al. (1 995>> und 31 kD (Doig et
al. (1 995>>, sowie uber Eisen-regulierte aussere Membranproteine mit den
Molekulargewichten 48 kD 50 kD
und 77 kD (Worst et al. (1995>>, Erythrocyten-bindende Antigene mit den
Molekulargewichten 25 kD und 59 kD (Huang et al. (1992>> und
Bindungsproteine fur Laminin, Kollagen 1 und IV, Fibronectin und
Vitronectin (Kondo et al. (1993>>. Weiterhin sind Proteine mit
Molekulargewichten von 19 kD (Drouet et al. (1991>>, 50 kD (Exner et
al., (1 995>> und 30 kD (Bolin et al. (1 995>> sowie ein Lipoprotein
mit 20 kD (Kostrzynska et al.
(1994>> und stammspezifische, oberflachenlokalisierte Antigene von 51 kD,
60 kD und 80...

...6 kD (Doig et al. (1 992>>, fur die Porine mit den Molekulargewichten
von 48 kD , 49 kD , 50 kD , 67 kD (Exner et al. (1995>>3 30 kD
(Tufano (1 994>> und 31 kD (Doig et al. (1 993>> und fur das 50
kD-Protein (Exner et al.

(1995...

...1998) als ein Blutgruppenantigen-bindendes Antigen, Bab A,
identifiziert. Allerdings konnte mit den bisher aus **Helicobacter
pylori** isolierten und charakterisierten Proteinen kein zufriedenstellend
wirksamer Impfstoff entwickelt werden.

Somit liegt der vorliegenden Erfindung...al. (1986) monospezifische
Antikörper zu isolieren. Eine Western Blot-Analyse gegen ein
Ganzkeim-Lysat von **Helicobacter pylori** zeigte, dass die
monospezifischen Antikörper gegen den Clon, der fur das Helikase-homologe
Protein...

...ein Gen von ureA beinhaltet. Dieses erkennt im Western-Blot eine
deutliche Proteinbande bei ca. 30 kD , was der Urease A-Untereinheit
zugeordnet werden kann. Der Nachweis der monospezifischen Antikörper
erfolgte mit...

4/3,KWIC/11 (Item 10 from file: 349)
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00510031 **Image available**

ANTIGEN LIBRARY IMMUNIZATION
IMMUNISATION PAR BIBLIOTHEQUE D'ANTIGENES

Patent Applicant/Assignee:

MAXYGEN INC,
Inventor(s):
PUNNONEN Juha,
BASS Steven H,
WHALEN Robert Gerald,

HOWARD Russell,
STEMMER Willem P C,
Patent and Priority Information (Country, Number, Date):
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Priority Application: US 9821769 19980211; US 9874294 19980211; US
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FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU
LV MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT UA
UG UZ VN YU ZW GH GM KE LS MW SD SZ UG ZW AM AZ BY KG KZ MD RU TJ TM AT
BE CH CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE BF BJ CF CG CI CM GA
GN GW ML MR NE SN TD TG
Publication Language: English
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Detailed Description

Detailed Description

... em5 I X 9 2 5 7 8 1 MSMEU 9 U M.smegmatis genTfor . 32 kDa protein
(partial)
emj5jX92572jMrEX26Q M.terrae gene tor 32 Rua protein U.66
(partial)
emb IX9256SIMSURU149 M.scrofulaceum gene tor 32 kDa protein 4 U
(par ...

eMbJZJJ6b6jMUJ2KP114 M.goraonae (ATUL 1447U) gene for 32kDa- 4 U
pro...

...78 nucieoproEe-in .38 2.6::]
RNA (...

Example 4

Evolution of Broad-spectrum Vaccines against **Helicobacterpylori**
Chronic infection of the gastroduodenal mucosae by **Helicobacterpylori**
bacteria is responsible for chronic active gastritis, peptic ulcers, and
gastric cancers such as
SUBSTITUTE...

4/3,KWIC/17 (Item 16 from file: 349)
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00436968 **Image available**
HELICOBACTER PYLORI DIAGNOSTICS
DIAGNOSTICS DE L'HELICOBACTER PYLORI

Patent Applicant/Assignee:

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NL PT SE
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Detailed Description

Detailed Description

... in the circulation. However,
such tests can suffer from a lack of specificity since
H. **pylori** appears to be antigenically cross-reactive

with Campylobacter jejuni and C. coli.

U.S. Patent...

...two antigen

components having molecular weights of 19.5 kDa, 26.5 kDa or 30 kDa, or alternatively, any one antigen component corresponding to a molecular weight of 35 kDa, 89 kDa, 116 kDa or 180 kDa. It is postulated by the inventors that the 19.5 kDa protein is a ferritin like protein, the 26.5 and 30 kDa proteins are ureases, the 89 kDa protein is VacA, and that the 116 kDa protein is CagA. The 35 kDa and 180 kDa were uncharacterized.

Finally, European Patent Publication 329,570, published 23 August 1989, describes immunoassays for H. pylori infection using pooled suspensions of sonicates of several H. pylori strains, as well as immunoassays using purified H. pylori flagellae.

Although faster and more sensitive than bacterial culture, antibody detection tests, such as those...

...above, can give false positive and negative results and generally do not distinguish between H. pylori Type I and Type II infection. Thus, an additional test must be conducted to determine whether the infection is due to H. pylori Type I or Type II.

Accordingly, the wide spread availability of an accurate and efficient assay for H. pylori infection that readily distinguishes between Type I and Type II infection, would be important

4/3,KWIC/21 (Item 20 from file: 349)

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00414241

PROTEINS, IN PARTICULAR MEMBRANE PROTEINS, OF HELICOBACTER PYLORI, THEIR PREPARATION AND USE

PROTEINES, NOTAMMENT PROTEINES MEMBRANAIRES, D'HELICOBACTER PYLORI, LEUR PREPARATION ET UTILISATION

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Detailed Description

Detailed Description

... the SEQ ID NO: 17 according to Table 1c has a molecular weight of approx. 25 kD, the protein containing a peptide sequence having the SEQ ID NO: 18 according to Table 1c has a molecular weight of approx. 25 kD, and the protein containing a peptide sequence having the SEQ ID NO: 19 according to Table 1c has a molecular weight of approx, 17 kD, The generally available H. pylori strain No. ATCC 43504 is used, for example, as the starting material when isolating the...and 15 were isolated and are specified as SEQ ID NOS: 20 (catalase), 24 (50 kD membrane protein), 25 (42 kD protein), 26 (36/35/ 32 kD protein) and 23 (28 kD protein). The gene coding for Hop C could not be isolated using oligonucleotide 7. However...

...Another approach is given by the recent access to the complete genomic sequence of H. pylori on the internet which allowed, for example, the identification of SEQ ID NO: 27.

The...NO: 25.

The protein has no signal sequence. The encoding region of SEQ ID NO: 25 codes for a protein of 399 amino acids with a molecular weight of 43.6 kD and an isoelectric point of 5,0, A search for homologous sequences using the BLASTP...

...al., 1990,, J. Mol, Biol. 215, 403-410) identified the 42 kD antigen of H. pylori as the elongation factor TU. The maximum percentage of identity (89%) was found with the...C) STANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULAR TYPE: Genomic DNA (iii) ORIGIN OF ORGANISM: Helicobacter pylori Direct experimental origin (iv) NAME OF CELL LINE: ATCC 43504 (v) FEATURES: from 344 to 520 bp signal peptide from 521 to 1507 bp mature protein (vi) PROPERTIES: 36/35/ 32 kD protein from Helicobacter pylori GATCGCTCTT TGAGTGATTC CTGTATTGCG TTTATTGGCA AACTCTTCGC CAAACATTTT 60 CTTACATTA GGGAAAATTA CCCCATCAAA AAACAAGTAG CCAATAAAAA TAATGGCGCA 120 CAATAAATGA...

4/3,KWIC/30 (Item 29 from file: 349)

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00318765

HELICOBACTER PYLORI ANTIGENIC PROTEIN PREPARATION AND IMMUNOASSAYS PREPARATION A BASE DE PROTEINE ANTIGENE D'HELICOBACTER PYLORI ET DOSAGE IMMUNOLOGIQUE

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GE HU IS JP KE KG KP KR KZ LK LR LT LU LV MD MG MN MW MX NO NZ PL PT RO
RU SD SE SG SI SK TJ TM TT UA UG US UZ VN KE MW SD SZ UG AT BE CH DE DK
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TG

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Detailed Description

Claims

English Abstract

A **Helicobacter pylori** protein preparation depleted of H. **pylori** antigens to which immunoreactivity is detected in H. **pylori** negative individuals and immunoassays using the protein preparation are described. The protein preparation is depleted of H. **pylori** antigens less than 30 kDa , especially 24 to 25 kDa and/or 18 to 19 kDa antigens.

French Abstract

La presente invention concerne une preparation a base de proteine d' **Helicobacter pylori** obtenue par depletion d'antigenes de H. **pylori** contre lesquels il a ete decele une immunoreactivite chez des individus H. **pylori** -negatifs. L'invention concerne egalement des dosages immunologiques utilisant cette preparation proteique. La preparation est obtenue par depletion d'antigenes de H. **pylori** inferieurs a 30 kDa , en l'occurrence d'antigenes de 24 a 25 kDa et/ou de 18 a 19 kDa .

Detailed Description

... Blaser,

1991 1 7)

Statements of Invention

According to the invention there is provided a

Helicobacter pylori protein preparation depleted of H.

pylori antigens to which immunoreactivity is detected in H. **pylori** negative individuals.

In a preferred embodiment of the invention, the immunoreactivity is antibody based.

In one particularly preferred embodiment of the invention the protein preparation is depleted of H.

pylori antigens characterised by a molecular weight less than 30 kDa . The protein preparation may be depleted of H. **pylori** antigens characterised by a molecular weight of approximately 24 to 25 kDa or derivative or fragment or precursor or mutant thereof.

In a preferred embodiment of the...

Claim

... presence of anti-H. **pylori** IgG antibodies. The figure shows a Western blot of H.

pylori probed with serum obtained from CLO negative individuals. All sera were diluted 1:100 in...

...pylori

(track B), or E. coli (track C). Fig. 3 : Partial purification of 18 and 25 kDa .

proteins : Both proteins were purified from whole **Helicobacter pylori** on the basis of molecular weight using preparative continuous-elution SDS-PAGE on a Model...

...Fig. 4 Sera obtained from CLO negative children

screened for the presence of anti-H. **pylori** IgG antibodies. The figure shows a Western blot of H. **pylori** probed with serum obtained from CLO negative children. All sera were diluted 1:50 in...

...serum

sample. Fig. 5 Antigens recognised on C, jejuni and E. coli by anti-H. **pylori** antiserum. The figure shows a Western blot of H. **pylori** (track A) I C. jejuni (track B) and E. coli '35 (track C) probed with rabbit anti-H. **pylori** antiserum. Each bacterium (5 gg) was subjected to SDS-PAGE followed by immunoblotting.

Fig. 6 Western blot of purified 25 kDa protein developed with serum from an individual negative for H. **pylori**. Purified 25 kDa protein was subjected to SDS-PAGE and Western blotting. The blot was probed with serum obtained from a subject uninfected with H. **pylori**.

Fig. 7 Biotinylation of proteins located on the surface of *Helicobacter pylori*. Agar-grown H. **pylori** were harvested in phosphate buffered saline (pH 7-3) and washed twice in this buffer...

...peroxidase (Sigma).

The present invention relates to improving the reliability of diagnostic immuno-assays for *Helicobacter pylori*. Previous studies (reference 3) have indicated a high level of false positive results...

...to improving the

reliability of serum, saliva or other mucous secretion based immunoassays for H. **pylori**. The present invention improves the specificity of immunoassays based on protein 'mixtures' for H. **pylori** by removing the 19 and kDa proteins from the protein mixture. As an example, removal of these proteins by preparative SDS PAGE analysts of all proteins less than 30 kDa from an antigenic preparation of H. **pylori** is cited. However, removal of such proteins might also be achieved by using affinity chromatography...

...underestimated. Western

blotting techniques were used to investigate antigen specificity of systemic responses to H. **pylori** in both healthy and H. **pylori** -infected individuals. The incidence of seropositivity in H. **pylori** negative individuals which has been shown is much greater than has previously been demonstrated. Furthermore, we have demonstrated that antibodies to a 25 kDa protein are detectable in the majority of H. **pylori** negative individuals. These were detected using a technique which we have modified called Enhanced Chemiluminescence...

...analysis reveals that the majority of

uninfected individuals have antibodies which are specific for H. **pylori** and recognise antigens which are not present on other micro organisms. Of these antigens the most common one recognised is a 25 kDa protein which appears to be specific to H. **pylori**. A second protein was also identified at 18 kDa in a large subgroup of H...

...of immuno-assay by removing antigens likely to result in false-positive analyses in H. **pylori** negative individuals.

n antigen component is present,, for the purposes of this invention, if...or microplates, or any other surface suitable for conducting an immunoassay.

Antigen components of H. **pylori** useful in this invention may be either covalently or non-covalently ("passively") bound to the...

...the formation of a complex between antibody in a serum or secretion sample and H. **pylori** ' antigens. In this particular case, the H. **pylori** antigen preparation is depleted of 18 to 19 and 24 to 25 **kDa** antigens. This is achieved by depletion of all antigens less than 30 **kDa** . Some form of detecting means is therefore necessary to identify the presence (or, if required...

...reporter molecule, and which is specific for at least part of the class of H. **pylori** -specific antibody found in the secretion.

ELISA

Immunoassays such as immunofluorescence assays (IFA), enzyme linked...react with each primary antibody, making the primary antibody more detectable. This system using H. **pylori** antigen depleted of 18 and 25 **kDa** proteins can utilise any substrate for binding of the antigens preparation and can use any...from which the 18kDa protein and other antigens to which immunoreactivity is detected in H. **pylori** negative individuals- has been removed. Removal of a strongly immunogenic antigen to which antibodies are present in H. **pylori** negative individuals should increase the discriminatory capabilities of ELISA in identifying people with active infection.

It is an object of the current invention to provide other purified proteins of H. **pylori** to which constitutive antibodies are detected in H. **Pylox-i** negative individuals.

It is also an object of the present patent to provide a H. **pylori** protein preparation from which proteins below 30 **kDa** have been removed on the basis for immunoassay for H. **pylori** .

We have developed a novel assay for detection of antibodies to H. **pylori** . This assay uses Western blotting and Enhanced Chemiluminescence (ECL). Using this assay we have demonstrated that approximately 75% of individuals who are negative for H. **pylori** by routine testing such as the rapid urease test have in fact got detectable antibodies to H. **pylori** (Fig. 1). Furthermore, these antibodies are not absorbed by C. jejuni or by E. Coli...

...ECL Western

blotting. Sera from n-infected individuals recognise a range of antigens on H. **pylori** . The most common antigen recognised is a 25 **kDa** protein which is recognised in over 70% of individuals who are negative for the organism on Rapid urease testing. Hence this suggests that the 25 **kDa** protein may be an immunodominant antigen which evokes a powerful immune response in individuals who...

...A second protein was

identified at 18 kDa which elicited significant antibody responses in H. **pylori** -negative children.

METHOD SECTION

Methods used in the identification and partial purification of two novel antigens from **Helicobacter pylori**.

Methods

Western Blotting. Proteins from SDS-PAGE gels (30% T/
2.67% C...and then exposed to X-ray film as described above.
Partial Purification of 18 and 25 kDa Proteins
Both proteins were partially purified from whole Helico
bacter **pylori** on the basis of molecular weight (Fig. 2)
using preparative continuous-elution sodium dodecyl
sulphate...

...quantitatively of preparative amounts of
proteins in a soluble form.

Purification Method

25 mg H. **pylori** were precipitated with ice-cold acetone,
washed once in acetone and the precipitate then
solubilised...

...the selected

proteins are detailed in the Methods section. Using
these exact conditions the 18 kDa proteins eluted
between 11-14 ml and the 25 kDa protein eluted within
16-20 ml. The molecular weights of the ...Western blot analysis of
antibody
response to H. pylori in individual negative for H.
pylori on Rapid urease testing. Western blotting was
performed as previously described using an enhanced
chemiluminescence...

...a

large range of H. pylori proteins were seen in
individuals who are H. **pylori** negative on Rapid urease
testing. The most common antigen to which an antibody
was detected with the 25 kDa protein. Figure 3 shows a
preparative SDS gel elution profile of the 25 kDa and 18
kDa proteins.

Detailed Description

Materials & Methods

Materials. All antibodies were obtained from Dako Ltd.,
High Wycombe...

...essentially as described by Laemmli (1970) 18 . A total of
mg of acetone-precipitated H. **pylori** protein were
- 19

located into each well. Gels were either stained with
Coomassie Blue R...

...Bio-Rad Laboratories, 3300 Regatta Blvd.,, Richmond,, CA
94804. The molecular masses are expressed as kDa .
Western Blotting. Proteins from SDS-PAGE gels (30 % T/
2.67% C) were electroblotted (0.8 MA/CM2 for 1 h) to PVDF...Each of these
subjects was CLO
negative, yet 83% had detectable antibodies (IgG) to H.
pylori (Fig. 1). Taken together, these data suggest
extensive prior contact with H. **pylori** . The most common
antigen to which an antibody was detected was a 25 kDa
species.

CLO negative children

The systemic humoral immune response (IgG) to H. **pylori**
was studied in two groups of children also. None of
these subjects had received any form of anti-H. **pylori**
therapy. However, in almost all cases the children had
a specific antibody response to H. **pylori** . The first
cohort studies consisted of twenty children (age range:
4 - 15 years), negative for H. **pylori** on CLO test. Of
these, 75% had detectable IgG antibodies to H. **Pylori**
(Fig. 4),

The second cohort of children (n = 20) were asymptomatic
and presented in hospital...

...prior contact with the bacterium,, when compared to the
considerably stronger response observed with H. **pylori**

positive individuals.

EXAMPLE 2

Cross Reactivity with other Bacteria

As many bacteria share common antigenic...

...addition to

E. coli, using two complimentary approaches. Firstly, the ability of the anti-H. **pylori** polyclonal antiserum to recognise antigens on both C. jejuni and E. coli was examined by Western blotting (Fig. 2).

Anti-H. **pylori** antiserum recognized a number of antigenic determinants on both E. coli and C. jejuni. Specifically, the antiserum recognises proteins of molecular mass 72, 50, 40, 36, and 25 kDa on C. jejuni and proteins of molecular mass 200, 116f 45, and 38 kDa on E. coli (Fig. 5). Of these, only 3 proteins (70, 25 kDa from C. jejuni and 200 kDa from E. coli) show pronounced cross-reactivity with anti-H. **Pylori** antiserum. Therefore, the observed cross reactivity is clearly not extensive. Secondly, absorption experiments demonstrated that...

...antigen

recognition was of mi

nor significance. Serum samples absorbed with clinical isolates of H. **pylori** and C. jejuni in addition to a commercially available strain of E. coli demonstrated that seroreactivity could be eliminated by absorbing with H. **pylori** but not with C. jejuni or E. coli (Fig. 2). Figure 2 is a representative...

...serum samples

screened in this study with similar results to those shown. The 18 and 25 kDa proteins were also detected in H. **pylori** Reference Strains NTCC 11637 and 11638 in addition to all clinical strains tested. Having partially...

...is a

representative experiment where the blot was incubated with the serum from an H. **pylori** un-infected individual. Clearly, this serum sample contains antibodies that specifically recognise the 24-26...estimated. A final protein concentration of 5 @ig/ml is required for the test. H. **pylori** antigens were purified on preparative SDS polyacrylamide gel electrophoresis and protein having a molecular weight of less than 30 kDa were removed. A final protein concentration of 5 pg/ml is required for the test.

Both antigenic preparations were aliquoted and stored at -70'C until required.

H. **pylori** antigens were diluted with bicarbonate buffer and 100 μ l of diluted antigens dispensed into each...as the cut-off point.

EXAMPLE 4 - Immunoassay

An example is provided whereby depletion of 25 and 19 kDa proteins,, in this case by elimination of proteins less than 30 kDa...

...bacterial culture performed on histological specimens.

The use of protein preparations depleted of proteins <30 kDa permitted a significantly increased specificity for the assay without influencing sensitivity.

Table 1

Anti-H. **pylori** antibodies IgG levels in patients with "CLO" test positive or negative individuals. IgG levels were measured by H. **pylori** antigen from whole bacterium. Patients were designated as H. **pylori** positive on the

basis of "CLO" test.
10/42 HP+ ve on "CLO" test 32...

...100%) 12/32 (38%)

Serology -ve 0/10 20/32 (62%)

Table 2

Anti-H. **pylori** antibodies IgG levels in patients with "CLO" test positive or negative individuals. IgG levels were measured by H. **pylori** antigen from protein greater than 30 kDa. Patients were designated as H. **pylori** positive on the basis of "CLO" test.

10/42 HP + ve on "CLO" test 32...

...will be appreciated that while we have referred to a molecular mass of 24 to 25 kDa and 18 to 19 kDa the molecular mass may lie in the 24-26 kDa and 17-19 kDa range.

Partial sequencing of the two antigens from **Helicobacter** PY1 0.ri

N-terminal sequence analysis

Purified 18 and 24 kDa proteins were electroblotted...

...degradation procedure as described by Matsudaira (1989)".

The N-terminal amino acid sequence of the 25 and 18 kDa protein are given in Sequence Id Nots 1 and 2 respectively.

Peptide Mapping

The N...and thus prevents N-blocking.

Amino acid sequences for internal peptides from the 18 and 25 kDa protein are given in Sequence Id. No.'s 3 and 4 respectively.

Extraction of **Helicobacter pylori** chromosomal DNA
Chromosomal DNA was extracted as described (Silhavy et al., 1984. Experiments with gene...Ala-Thr-Glu-Glu
30 35 40

Ile-Tyr-Glu-Glu

45

CLAIMS

A H. **pylori** protein preparation depleted of H. **pylo.ri** antigens to which immunoreactivity is detected in H...

...or 2 depleted of H. **pylori** antigens
characterised by a molecular weight less than 30 kDa .

4 A H. **pylori** protein preparation as claimed in any of claims 1 to 3...

...H. **pylori**
antigens characterised by a molecular weight less than 27 kDa.

7 A H. **pylori** protein preparation as claimed in any preceding claim depleted of antigens characterised by a molecular weight of approximately 24 to 25 kDa or derivative or fragment or precursor or mutant thereof,

8 A H. **pylori** protein preparation as claimed in any preceding claim depleted of antigens characterised by a molecular...

...18 to 19 kDa or derivative or
fragment or precursor or mutant thereof.
A H. **pylori** protein preparation as claimed in any preceding claim depleted of :- I
(i) antigens characterised by a molecular weight of approximately 24 to 25 kDa or

derivative or ...approximately 18 to 19 kDa or fragment or precursor or mutant thereof.

10 A H. **pylori** protein preparation as claimed in any of claims 7 to 9 in which the 24 to 25 kDa antigen is further characterised in that it includes an N-terminal amino acid sequence shown in Sequence Id. No. 1 or portions thereof.

11 A H. **pylori** protein preparation as claimed in any of claims 7 to 10 in which the 25 kDa antigen is further characterised in that it includes an internal amino acid terminal sequence shown in Sequence Id. No. 4 or portions thereof.

12 A H. **pylori** protein preparation as claimed in claims 8 to 11 in which the 18 to 19...

...amino acid sequence shown in Sequence Id. No. 2 or portions thereof.

13 A H. **pylori** protein preparation as claimed in any of claims 8 to 12 in which the 18...

4/3,KWIC/31 (Item 30 from file: 349)

DIALOG(R)File 349:PCT FULLTEXT

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00318764

HELICOBACTER PROTEINS AND VACCINES
PROTEINES D'HELICOBACTER ET VACCINS

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Detailed Description
Claims

English Abstract

A vaccine includes at least one **Helicobacter**, especially **Helicobacter pylori** protein to which immunoreactivity is detected in H. **pylori** negative individuals. The **Helicobacter** proteins are preferably less than 30 kDa and the vaccine especially includes 24 to 25 kDa and/or 18 to 19 kDa proteins. The vaccine may include interleukin (12) as an adjuvant.

French Abstract

La presente invention concerne au moins une proteine d' **Helicobacter**, en

l'occurrence une proteine d' **Helicobacter pylori** contre laquelle une immunoreactivite a ete detectee chez des individus H. **Pylori** -negatifs. Les proteines d' **Helicobacter** sont de preference inferieures a 30 kDa , et le vaccin comprend des proteines de 24 a 25 kDa et/ou de 18 a 19 kDa . Le vaccin peut comporter de l'interleukine 12 comme adjuvant.

Detailed Description

... immunoreactivity is antibody based.

In a preferred embodiment of the invention, the protein is a **Helicobacter pylori** protein.

In a preferred embodiment of the invention the protein has a molecular weight of less than 30 kDa , especially less than 29 kDa , particularly less than 28 kDa and ideally less than 27 kDa .

In a particularly preferred embodiment of the invention, the vaccine includes a 24 to 25 kDa protein or a derivative or fragment or precursor or mutant thereof, The 24 to 25 kDa protein is further characterised in that it has a N-terminal amino acid sequence listed in Sequence Id. No. 2, or a portion thereof. The 24 to 25 kDa protein is further characterised in that it has an internal amino acid sequence listed in...

...include a peptide delivery system.

The vaccine is ideally for the treatment or prophylaxis of **Helicobacter pylori** infection or **Helicobacter pylori** associated disease(s).

According to another aspect of the invention...

...in H. **pylori** negative individuals. Preferably, the immunoreactivity is antibody based.

Preferably the **Helicobacter pylori** is a **Helicobacter pylori** protein.

In a preferred embodiment of the invention, the protein has a...

...and ideally less than 27 kDa.

In a particularly preferred embodiment of the invention, the **Helicobacter pylori** protein is a 24 to 25 kDa protein or derivative or fragment or precursor or mutant thereof.

The 24 to 25 kDa **Helicobacter pylori** protein is characterised in that it includes the N-terminal amino acid sequence listed in Sequence Id, No, 2, or a portion thereof.

The 24 to 25 kDa **Helicobacter pylori** protein is further characterised in that it includes an internal amino acid sequence listed in...

...Id. No. 4, or a portion thereof.

In another preferred embodiment of the invention, the **Helicobacter pylori** is an 18 to 19 kDa protein or derivative or fragment or precursor or mutant thereof, The 18 to 19 kDa **Helicobacter pylori** is characterised in that it includes the N-terminal amino acid sequence listed in Sequence Id. No, 1, or a portion thereof.

The 18 to 19 kDa **Helicobacter pylori** is further characterised in ...of the invention for the preparation of a medicament for the treatment or prophylaxis of **Helicobacter pylori** associated disease(s).

The invention further provides monoclonal or polyclonal antibodies or fragments thereof...

...and
antibodies in the treatment or prophylaxis of **Helicobacter** associated disease(s) and in particular **Helicobacter pylori** associated disease(s).

The invention also provides a vaccine for the treatment or prophylaxis of **Helicobacter pylori** associated disease comprising an immunogenically effective amount of the 24 to 25 kDa **Helicobacter pylori** protein and/or the 18 to 19 kDa **Helicobacter pylori** protein of the invention, an adjuvant such as Interleukin 12, and an antibiotic. The vaccine...

...invention also includes the use of interleukin 12 in combination 'with the 18 to 19 kDa protein, the 24 to 25 kDa or any other H. pyloz-i subunit as an adjuvant therapy.

Therefore, in another aspect,, the invention provides a vaccine against H. **pylori** comprising an immunagenically effective amount of a **Helicobacter**- or a subunit, fragment, derivative, precursor or mutant thereof in combination with interleukin 12 as an adjuvant.

Preferably the **Helicobacter** is **Helicobacter pyloz-i**.

In one embodiment of the invention the vaccine includes an antibiotic and may...

...of Drawinas
Fig. 1 Adult sera (CLO negative) screened for the presence of anti-H. **pylori** IgG antibodies. The figure shows 'a Western blot of H, **pylori** probed with serum obtained from CLO negative individuals. All sera were diluted 1:100 in...track B), or E. coli (track C),
Fig. 3 Partial purification of 18 and 25 kDa proteins : Both proteins were purified from whole **Helicobacter pylori** on the basis of molecular weight using preparative continuous-elution SDS-PAGE on a Model...

...Fig. 4 Sera obtained from CLO negative children screened for the presence of anti-H. **pylori** IgG antibodies. The figure shows a Western blot of H. **pylori** probed with serum obtained from CLO negative children. All sera were diluted 1:50 in...

...serum
sample.

Fig. 5 Antigens recognised on C. jejuni and E. coli by anti-H. **pylori** antiserum. The figure shows a Western blot of H. **pylori** (track A) , C. jejuni (track B) and E. coli (track C) probed with rabbit anti-H. **pylori** antiserum, Each bacterium (5 gg) was subjected to SDS-PAGE followed by immunoblotting.

Fig. 6 Western blot of purified 25 kDa protein developed with serum from an individual negative for H. pylori. Purified 25 kDa protein was subjected to SDS-PAGE and Western blotting. The blot was probed with serum obtained from a subject uninfected with H. pylori.

Fig. 7 Biotinylation of proteins located on the surface of Helicobacter pylori. Agar-grown H. pylori were harvested in phosphate buffered saline (pH 7.3) and washed twice in this buffer...00036

approach we use Western blotting to investigate antigen specificity of systemic responses to H. pylori in both healthy and H. pylori-infected individuals and shown that the incidence of seropositivity in H. pylori negative individuals is much greater than has previously been demonstrated. Furthermore, we have demonstrated that antibodies to a 24 to 25 kDa protein are detectable in the majority of H. pylori negative individuals.

These were detected using a technique which we have modified called Enhanced Chemiluminescence...

...analysis reveals that the majority of uninfected individuals have antibodies which are specific for H. pylori and recognise antigens which are not present on other micro organisms. Of these antigens the...

...one recognised is a 24 to kDa protein which appears to be specific to H.

Pylori. Hence, these data suggest that immunisation with the 24 to 25 kDa protein or sub-unit thereof could have the potential to confer protective immunity on individuals...was also identified at 18 to 19 kDa in a large subgroup of H.

pylori negative individuals. Similarly, immunization with this protein or subunit thereof could also confer protective immunity.

We have developed a novel assay for detection of antibodies to H. pylori. This assay uses Western blotting and Enhanced Chemiluminescence (ECL). Using this assay we have demonstrated that approximately 75% of individuals who are negative for H. pylori by routine testing such as the rapid urease test have in fact got detectable antibodies to H. pylori (Fig. 1).

Furthermore, these antibodies are not absorbed by C, jejuni or by E. coli...

...ECL Western

blotting. Sera from un-infected individuals recognize a range of antigens on H. pylori. The most common antigen recognised is a 24 to 25 kDa protein which is recognised in over 70% of individuals who are negative for the organism on Rapid urease testing. Hence this suggests that the 24 to 25 kDa protein may be an immunodominant antigen which evokes a powerful immune response in individuals who...

...protein was identified at 18 to 19 kDa which elicited significant antibody responses in H. pylori-negative children. These proteins have been further

characterised by N-terminal and internal sequencing as...

...in response to antigen. As stated previously, antigen-specific interferon production is reduced with H. *pylori* positive individuals. The addition of IL-12 to immunisation schedules with a 25 kDa protein would be expected to boost host immunity to H. *pylori* by augmenting the '@'@interferon response.

Materials. All antibodies were obtained from Dako Ltd,, High Wycombe...

...19 essentially as described by Laemmli (1970) A total of mg of acetone-precipitated H. *pylori* protein were located into each well. Gels were either stained with Coomassie Blue R-250...

...Bio-Rad Laboratories, 3300 Regatta Blvd., Richmond,. CA 94804. The molecular masses are expressed as kDa .

Western Blotting Proteins from SDS-PAGE gels (30 % T/2.67% C) were electroblotted (0.8 mA/CM² for 1 h) to...and then exposed to X-ray film as described above.

Partial Purification of 18 and 25 kDa Proteins Both proteins were partially purified from whole *Helicobacter pylori* on the basis of molecular weight (Fig. 2) using preparative continuous-elution sodium 15- dodecyl...

...quantitatively purify preparative amounts of proteins in a soluble form. Purification Method 25 mg H. *pylori* were precipitated with ice-cold acetone, washed once in acetone and the precipitate then solubilised...

...once optimal electrophoretic conditions have been established. Preliminary optimization protocols entailed electrophoresing mixtures of H. *pylori* proteins under conditions designed to favour high resolution of low molecular weight proteins. The f...a large range of H. *pylori* proteins were seen in individuals who are H. *pylori* negative on Rapid urease testing. The most common antigen to which an antibody was detected with the 25 kDa protein. Figure 3 shows a preparative SDS gel elution profile of the 25 kDa and 18 kDa proteins. These proteins have been further characterised by N-terminal and internal sequencing as outlined...

...CLO negative adults Similarly, a cohort of 19 adult sera was screened for anti-H. *pylori* IgG antibodies. Each of these subjects was CLO negative, yet 83% had detectable antibodies (IgG) to H. *pylori* (Fig, 1). Taken together, these data suggest extensive prior contact with H. *pylori* . The antigen to which an antibody was detected most common an was a 25 kDa species.

CLO negative children

t

The systemic humoral immune response (IgG) to H. *pylori* was studied in two groups of children also. None of these subjects had received any form of anti-H. *pylori* therapy. However, in almost all cases the children had a specific antibody response to H. *pylori* . The first

cohort studies -consisted of twenty children (age range.

4 - 15 years), negative for *H. pylori* on CLO test. Of these,, 75% had detectable IgG antibodies to *H. pylori* (Fig. 4),

The second cohort of children (n = 20) were asymptomatic and presented in hospital...

...addition to'

E. coli, using two complimentary approaches. Firstly, the ability of the anti-*H. pylori* polyclonal antiserum PCT1IE95/00036 - 17

to recognise antigens on both *C. jejuni* and *E. coli* was examined by Western blotting (Fig. 2).

Anti-*H. pylori* antiserum recognized a number of antigenic determinants on both *E. coli* and *C. jejuni*, Specifically, the antiserum recognises proteins of -molecular mass 72, 50, 40, 36, and 25 kDa on *C. jejuni* and proteins of molecular mass 200, 116, 45, and 38 kDa on *E. coli* (Fig. 5). Of these, only 3 proteins (70, 25 kDa from *C. jejuni* and 200 kDa from *E. coli*) show pronounced cross-reactivity with anti-*H. pylori* antiserum, 'Therefore, the observed cross reactivity is clearly not extensive. Secondly, absorption experiments demonstrated that...

...reactive antigen

recognition was of minor significance. Serum samples absorbed with clinical isolates of *H. pylori* and *C. jejuni* in addition to a commercially available strain of *E. coli* demonstrated that seroreactivity could be eliminated by absorbing with *H. pylori* but not with *C. jejuni* or *E. coli* (Fig. 2). Figure 2 is a representative...

...serum samples

screened in this study with similar results to those shown. The 18 and 25 kDa proteins were also detected in *H. pylori* Reference Strains NTCC 11637 and 11638 in addition to all clinical strains tested.

Having partially...T-helper 2 pathway.

Fig. 8 illustrates thymidine incorporation of lymphocytes in response to *H. pylori* in the presence and absence of interleukin 12. Interleukin 12 significantly augmented proliferation of peripheral...in the art that while we have referred to a molecular mass of 24 to 25 kDa and 18 to 19 kDa the molecular mass may lie in the '24-26 kDa and 17-19 kDa range. Other related organisms such as *H. Felis* or *H. mustelis* may produce gastric diseases in animal models.

Cross reactivity between proteins from *Helicobacter* species may' mean that antigens from an individual bacterial species could provide protection in an...

...which is not its normal host.

The dominant antigens to which antibody is detected in *Helicobacter pylori* -negative individuals are the 18-19 and 24- 25 kDa antigens. Hence,, use of an antigenic preparation containing all antigens less than 30 kDa , preferably less than 29, ideally less than 28 and preferably less than 27 kDa and would be enriched in the immunodominant antigens to be used in putative vaccine.

Partial sequencing of the two antigens from *Helicobacter* Rylori

N-terminal sequence analysis

Purified 18 and 24 kDa proteins were electroblotted to PVDF...

...degradation procedure

as described by Matsudaira (198921),

The N-terminal amino acid sequence of the 25 and 18 kDa protein are given in Sequence Id No's 1 and 2 respectively.

- 23

Peptide Mapping

The N-chlorosuccinimide peptide mapping method of Lischwe and Ochs (1982) 22 was...

...and thus prevents N-blocking.

Amino acid sequences for internal peptides from the 18 and 25 kDa protein are given in Sequence Id. No.'s 3 and 4 respectively.

Extraction of **Helicobacter pylori** chromosomal DNA
Chromosomal DNA was extracted as described (Silhavy et al., 1984. Experiments with gene...

Claim

A vaccine including at least one **Helicobacter** protein or derivative or fragment or precursor or mutant thereof to which immunoreactivity is detected in H. **pylori** negative individuals.

2 A vaccine as claimed in claim 1 wherein the immunoreactivity is antibody...

...3* A vaccine as claimed in claims 1 or 2, wherein the protein is a **Helicobacter pylori** protein.

4 A vaccine as claimed in any of claims 1 to 3 wherein the...

...a single protein or a mixture of proteins having a molecular weight of less than 30 kDa .

5 A vaccine as claimed in claim 4, wherein the protein has a molecular weight...

...claims 4 to 6,, wherein the protein has a molecular weight of less than 27 kDa .

8 A vaccine as claimed in any preceding claim, including a 24 to 25 kDa protein or a derivative or fragment or precursor or mutant thereof.

A vaccine as claimed...as claimed in any of claims 1 to 27 for the treatment or prophylaxis of **Helicobacter Pylori** infection or **Helicobacter Pyl Ori** associated disease.

29 A **Helicobacter** protein or derivative or...

...kDa protein or a derivative or fragment or precursor or mutant thereof,

37 A **Helicobacter pylori** protein as claimed in claim 36 wherein the 24 to 25 kDa protein, has an N-terminal amino acid sequence listed in Sequence Id, No. 1, or a portion thereof.

.38, A **Helicobacter pylori** as claimed in claim 36 or 37,, wherein the 24 to 25 kDa protein,, has an internal amino acid sequence listed in Sequence

Id. No. 4, or a portion thereof.

39 A **Helicobacter pylori** protein as claimed in any of claims 32 to 35 wherein the protein is an 18 to 19 kDa protein, or a derivative, fragment or precursor or mutant thereof.

40 A **Helicobacter pylori** protein as claimed in claim 38, wherein the 18 to 19 kDa protein...

...amino acid sequence
listed in Sequence Id. No. 2, or a portion thereof.

41 A **Helicobacter pylori** protein as claimed in claims 39 or 40, wherein the 18 to 19 kDa protein a portion thereof.

42 A **Helicobacter pylori** protein as claimed in claim 39 to 41 wherein the 18 to 19 kDa protein...
?t s3/3,kwic/5 19 44

3/3,KWIC/5 (Item 5 from file: 349)
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00970206

IMMUNOGENIC HELICOBACTER SURFACE PROTEINS AND THEIR USE
PROTEINE IMMUNOGENE DE SURFACE, DU GENRE HELICOBACTER ET LEURS UTILISATIONS

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Detailed Description

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Detailed Description

... Rad).

RESULTS

Protein profiles in 1-D SDS-PAGE.

Extracted surface proteins of the four **Helicobacter** spp were compared after separation by I D SDS-PAGE. Gels were stained with CBB various Ms and 18-22 of these were more intensively stained. Proteins of H. **pylori** and 11. pulloruni showed more similarities in their protein profiles compared with H. bilis and H. hepaticus. Fewer proteins were detected in extract of H. hepaticus (n= 23) and the major bands were clustered below 32 kDa (Figure 1).

I 0 2-DE protein profiles of H. pullorunt.

A total of 499...of antibodies was applied to evaluate the presence of nsp in samples of the four **Helicobacter** spp. No antibody reactivity was observed with the anti-Hsp47, anti-Bsp70 and anti-Hsp56 antibodies to any of the **Helicobacter** species.

By I -DE IB the anti-Hsp60 and anti-Hsp65 recognised proteins of H. pullorum (60 and 32 kDa) (data not shown). By 2-DE IB anti-Hsp60 reacted to 15 spots with mass...

3/3,KWIC/19 (Item 2 from file: 654)
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(c) Format only 2003 The Dialog Corp. All rts. reserv.

0005351371 **IMAGE Available
Derwent Accession: 2003-712672

US 20030166027

Compositions, test kits and methods for detecting helicobacter pylori

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	Publication Number	Kind	Date	Application Number	Filing Date
Main Patent	US 20030166027	A1	20030904	US 200280113	20020221

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Abstract:

Four proteins are obtained from H. **pylori** bacteria each of which has regions which act as antigens specific to H. **pylori** . The proteins are isolated, purified and designated HP1, HP2, HP3, and HP4 with respective molecular weights of 32 kd , 30 kd , 23 kd and 15 kd . An assay, a method and a kit is developed utilizing a combination of at least three of these protein to detect the presence of antibodies to H. **pylori** in human sera. The method of detection is quantified and suitable for monitoring the eradication of H. **pylori** bacteria by drug therapy of human patients infected by these bacteria...

Description of the Invention:

...0017] The composition according to the invention comprises at least three **Helicobacter pylori** derived proteins or their antigenic regions, wherein the proteins are selected from the group of **Helicobacter pylori** derived proteins which are identified by SDS PAGE to consist of antigens specific to **Helicobacter pylori** of molecular weights 32 kd ; 30 kd ; 23 kd ; and 15 kd . These antigens from H. **pylori** have not been used in this combination in other available tests. These proteins were assigned...

...0018] HP1 32 kd protein...on Mwt and iso-electric point in the database to identify the proteins of H. **pylori** containing the peptides. Four immuno-reactive spots were found in the low Mwt range; HP1 at 32 kDa with a pI of ~7.5, HP2 at 30 kDa with a pI of ~6.1; HP3 at 22 kDa with pI of ~8.9; and HP4 at 14 kDa with a pI of ~5...

Exemplary or Independent Claim(s):

...HP3 and HP4, each of said proteins comprising regions which act as antigens specific to **Helicobacter pylori**, HP1 having of molecular weight of 32 kd, HP2 having of molecular weight of...

...having of molecular weight of 23 kd, and HP4 having of molecular weight of 15 kd, each of said proteins being derived from **Helicobacter pylori** bacteria...

...26. A method of using a combination of at least 3 proteins from **Helicobacter pylori** for detecting the presence or absence of antibodies resulting from **Helicobacter pylori** infection wherein the proteins are selected from the group consisting of HP1, HP2, HP3 and HP4, each of said proteins comprising regions which act as antigens specific to **Helicobacter pylori**, HP1 having of molecular weight of 32 kd, HP2 having of molecular weight of 30 kd, HP3 having of molecular weight of 23 kd, and HP4 having of molecular weight of 15 kd, each of said proteins being derived from **Helicobacter pylori** bacteria.

3/3,KWIC/44 (Item 2 from file: 348)

DIALOG(R) File 348:EUROPEAN PATENTS

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00969625

HELICOBACTER PYLORI DIAGNOSTICS

HELICOBACTER PYLORI DIAGNOSTIKA

DIAGNOSTICS DE L'HELICOBACTER PYLORI

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SPEC B	(English)	200223	10200
Total word count - document A			0
Total word count - document B			13968
Total word count - documents A + B			13968

...SPECIFICATION in the circulation. However, such tests can suffer from a lack of specificity since **H. pylori** appears to be antigenically cross-reactive with **Campylobacter jejuni** and **C. coli**.
U.S. Patent...

...two antigen components having molecular weights of 19.5 kDa, 26.5 kDa or

30 kDa , or alternatively, any one antigen component corresponding to a molecular weight of 35 kDa , 89 kDa, 116 kDa or 180 kDa. It is postulated by the inventors that the 19.5 kDa protein is a ferritin-like protein, the 26.5 and 30 kDa proteins are ureases, the 89 kDa protein is VacA, and that the 116 kDa protein is CagA. The 35 kDa and 180 kDa were uncharacterized.

Finally, European Patent Publication 329,570, published 23 August 1989, describes immunoassays for H. pylori infection using pooled suspensions of sonicates of several H. pylori strains, as well as immunoassays using purified H. pylori flagellae.

Although faster and more sensitive than bacterial culture, antibody detection tests, such as those...

...above, can give false positive and negative results and generally do not distinguish between H. pylori Type I ...an additional test must be conducted to determine whether the infection is due to H. pylori Type I or Type II.

Accordingly, the wide spread availability of an accurate and efficient assay for H. pylori infection that readily distinguishes between Type I and Type II infection, would be important for...

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 00/02419

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/569 C07K14/205 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>ANDERSEN L P ET AL: "Isolation and Preliminary Evaluation of a Low-Molecular-Mass Antigen Preparation for Improved Detection of Helicobacter pylori Immunoglobulin G Antibodies."</p> <p>CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 2, no. 2, 1995, pages 156-159, XP000917860</p> <p>ISSN: 1071-412X</p> <p>abstract</p> <p>page 156, column 1, paragraph 2</p> <p>page 157, column 1, paragraph 1 -column 2, paragraph 1</p> <p>figure 1</p> <p>page 157, column 2, paragraph 5 -page 158, column 2, paragraph 2</p> <p style="text-align: center;">--- -/--</p>	5-25



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

I document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

A document member of the same patent family

Date of the actual completion of the international search

18 August 2000

Date of mailing of the international search report

12.09.00

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Montrone, M

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/02419

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>WO 96 26740 A (ENTERON LIMITED PARTNERSHIP) 6 September 1996 (1996-09-06) abstract page 6, line 25 -page 7, line 1 page 11, line 4 - line 16 page 19, line 21 - line 37 tables 3,4</p>	5-25
Y	<p>US 5 846 751 A (PAWLAK JAN WACLAW ET AL) 8 December 1998 (1998-12-08) abstract column 3, line 29 - line 34 column 5, line 17 - line 30 table 1</p>	5-25
Y	<p>AUCHER P ET AL: "Use of immunoblot assay to define serum antibody patterns associated with Helicobacter pylori infection and with H. pylori-related ulcers." JOURNAL OF CLINICAL MICROBIOLOGY, vol. 36, no. 4, April 1998 (1998-04), pages 931-936, XP000917831 ISSN: 0095-1137 abstract page 934, column 2, paragraph 2</p>	5-25
A	<p>NISHIZONO AKIRA ET AL: "Serological assessment of the early response to eradication therapy using an immunodominant outer membrane protein of Helicobacter pylori." CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 5, no. 6, November 1998 (1998-11), pages 856-861, XP000917721 ISSN: 1071-412X abstract page 857, column 2, paragraph 1 - paragraph 3 page 860, column 1, paragraph 2 -column 2, paragraph 3</p>	26

24

Table 4. Identification of the *H. pylori* antigens by mass spectrometry, recombinant expression and purification

Spot	Antigen	Relative Mwt kDa	pI	TIGR ID	recombinant t expression	C- or N- term 6xHis Tag +/- SP
HP1(a)	Neuraminyl-lactose- binding Hemagglutinin precursor HpaA	32	7.5	HP0797	+++	C-term - SP
HP1(b)	3-Oxoacid COA transferase subunit A CoA-trans	32	7.5	HP0691	+++	C-term no SP
HP2(a)	Elongations factor P EF-P	30	6.1	HP0177	+++	C-term no SP
HP2(b)	Peptidoglycan associated lipoprotein precursor Omp18	30	6.1	HP1125	+++	C-term - SP
HP3(a)	Adhesin-thiol peroxidase TagD	22	8.9	HP0390	+	C-term no SP
HP3(b)	Hypothetical protein HP0596	22	8.9	HP0596	+++	N-term - SP
HP4(a)	Ribosomal protein L7/L12 RPL7/L12	14	5.9	HP1199	+++	C-term no SP
HP4(b)	ATP Synthase F ₀ subunit b' ATP-F ₀ b'	14	5.9	HP1137	++	N-term - SP

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As is known in the art TIGR ID refers to identification from a widely available known genomic data base.

Table 5. Antigenic profile of the low Mwt recombinant antigens with *H. pylori* positive and negative sera.

2D Spot	Antigen N5	P1	P2	P3	P4	P5	P6	P7	P9	P10	N1	N2	N3	N4
HP1(a)	-	+	+	+	+	(+)	+	+	-	-	-	-	-	-
HP1(b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HP2(a)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HP2(b)	+	+	+	+	+	+	+	+	-	-	-	-	-	-
HP3(a)	-	+	+	+	+	+	+	+	-	-	-	-	-	-
HP3(b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HP4(a)	+	+	+	-	+	+	+	+	-	-	-	-	+	-
HP4(b)	-	(+)	-	-	-	-	-	-	-	-	-	-	-	-

Serological Assessment of the Early Response to Eradication Therapy Using an Immunodominant Outer Membrane Protein of *Helicobacter pylori*

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TOSHIHIRO KUBOTA,² MASARU NASU,² MAKOTO WATANABE,³
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Received 12 March 1998/Returned for modification 22 April 1998/Accepted 23 July 1998

Eradiation of *Helicobacter pylori* infection cures gastritis and prevents recurrence of peptic ulcers. Endoscopy is usually used to evaluate the effectiveness of eradication therapy. We designed a new noninvasive assay system for the early evaluation of eradication of *H. pylori* infection in which a crude *H. pylori* outer membrane protein preparation (HPOMP) is used as an antigen, and we determined the sensitivity and specificity of the serological assay system. Immunoblot analysis showed that anti-HPOMP antibodies reacted to a protein with a molecular mass of approximately 29 kDa. In those patients who responded to therapy, the anti-HPOMP immunoglobulin G (IgG) titers measured by enzyme-linked immunosorbent assay (ELISA) at 1 month after the end of therapy were significantly lower than those before treatment (34.8% reduction; $P < 0.001$), and the posttreatment reduction in the antibody titer was significantly greater than that of the titer measured with a commercially available anti-*H. pylori* IgG ELISA (34.8% versus 16.1%; $P < 0.001$). When a 25% reduction of anti-HPOMP IgG titer at 1 month after the end of treatment was taken as the cutoff value for *H. pylori* eradication, the sensitivity and specificity of our new assay were 75% (51 of 68 treatment responders) and 96% (22 of 23 nonresponders), respectively. Our results indicate that the novel serological test with HPOMP might be a clinically useful tool for assessment of eradication of *H. pylori*.

Helicobacter pylori is an important pathogen which causes gastritis, peptic ulcer, and intestinal metaplasia, and long-term infection with this organism is a risk factor for gastric carcinoma (11). Therefore, eradication of *H. pylori* is important, especially in patients with peptic ulcers (5). Apart from serological detection and the urea breath test (UBT), invasive tests involving endoscopy are the main methods for evaluation of the efficacy of eradication therapy. Although the currently available serological tests are convenient and the UBT offers a highly sensitive and specific means of detection of *H. pylori*, the former tests cannot detect reductions in antibody titer in the early posteradication period (8), while the latter test is expensive and not readily available to the majority of general practitioners, especially in Japan. Thus, a noninvasive and sensitive method that detects eradication of the organism is desirable.

In the present study, we describe the design and evaluation of a new serological assessment test for the eradication of *H. pylori* in which a crude *H. pylori* outer membrane protein preparation (HPOMP) is used as an antigen.

MATERIALS AND METHODS

Patients and sera. One hundred two patients (61 males and 41 females; mean age, 52.4 years; range, 13 to 76 years) were diagnosed with *H. pylori* infection in the Second Department of Internal Medicine between 1989 to 1996. The diagnosis was based on the following tests: bacterial culture, histopathological examination, and rapid urease test. The sample consisted of 38 patients with chronic gastritis, 27 with gastric ulcer, 36 with duodenal ulcer, and 1 patient with normal findings on endoscopic examination. All patients received a proton pump inhibitor or histamine blocker (H_2 blocker) combined with amoxicillin (1,500 mg/day)

or clarithromycin (400 to 800 mg/day) and metronidazole (500 mg/day) for 7 days. *H. pylori* was not detected by bacterial examination at 1 month after the end of eradication therapy in 68 patients (responders). *H. pylori* was not eradicated in the remaining 34 patients (nonresponders).

Blood samples were obtained just before treatment and at 1, 3, 6, and 12 months after the end of therapy. Among nonresponders, we were able to obtain serum samples from only 23 patients at 1 month after the end of therapy. Control sera used in this study were obtained from 19 individuals (10 males and 9 females; mean age, 38.9 years) who were negative for *H. pylori* infection by bacterial examination and from 23 newborn babies (14 males and 9 females). Each patient gave informed consent after receiving a full explanation of the purpose and design of the study.

Preparation of HPOMP. The *H. pylori* type strain ATCC 43504 was used for preparation of the antigen in the present study. *H. pylori* was grown on blood agar plates with 10% defibrinated sheep blood (GIBCO BRL, Grand Island, N.Y.) in an atmosphere of 10% CO₂ and 5% O₂ with CampyPak-Plus (BBL Microbiology Systems, Cockeysville, Md.). *H. pylori* was scraped and collected from plates and pulverized by a French press (12,000 lb/in², three times), and the particulate fraction was pelleted by ultracentrifugation at 200,000 × *g* for 3 h. The resulting whole particulate fraction was subjected to linear sucrose density gradient (SDG) separation from 25% to 65% (wt/wt). After centrifugation at 120,000 × *g* for 20 h, the gradient was divided from the bottom into six fractions. In order to identify the fraction containing the outer membrane, we determined the insolubility of each fraction with 1% *N*-lauroylsarcosine, the electrophoretic patterns of proteins detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the reactivities of electrophoresed proteins with anti-urease monoclonal antibody (kindly provided by Kumiko Nagata, Hyogo Medical University) detected by immunoblotting and by the presence of urease activity. After the outer membrane was identified by its insolubility with *N*-lauroylsarcosine, the fraction that contained the outer membrane proteins was pelleted by ultracentrifugation at 200,000 × *g* for 4 h. The resulting pellet was resuspended in an aliquot of membrane buffer consisting of 0.25 M sucrose, 50 mM triethanolamine, and 1 mM dithiothreitol and used as the crude HPOMP antigen.

ELISA and immunoblotting. The serum sample was subjected to two types each of enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB) analyses. The first ELISA was a conventional ELISA performed by using a commercial GAP immunoglobulin G (IgG) test (Biomerica, Newport Beach, Calif.) (Piliplate *Helicobacter* II; Fuji Rebio Inc., Tokyo, Japan). Determination of ELISA units (EU) was performed with 1:200-diluted test sera and an accompanying positive control serum according to the instructions provided by the manufacturer. In the second type of ELISA, HPOMP at 10-μg/ml concen-

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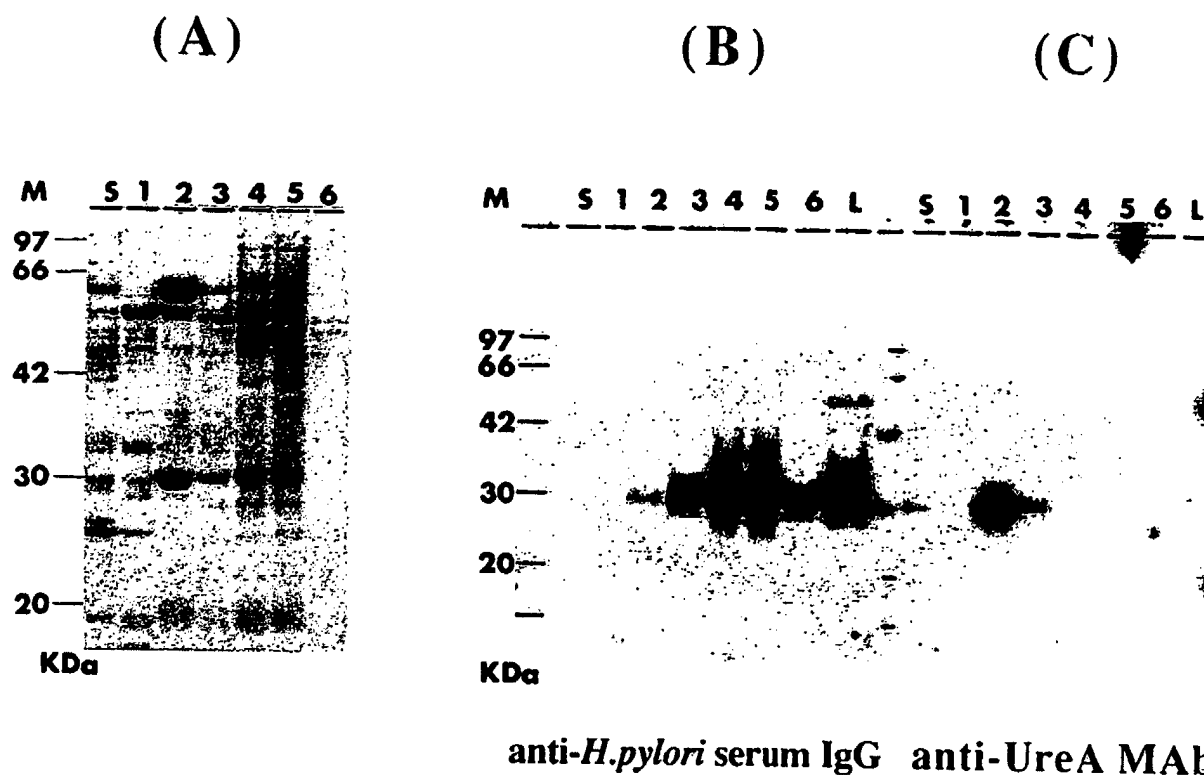


FIG. 1. SDS-PAGE profiles of HPOmp fraction and immunoreactivity by WB analysis. (A) SDS-PAGE patterns of soluble fraction (lane S) and membrane fractions (lanes 1–6) separated by 25% to 65% (wt/wt) linear SDG. (B and C) Immunoreactivities of protein A-Sepharose purified IgG obtained from an *H. pylori*-infected patient (B) and anti-urease monoclonal antibody (C) (kindly provided by K. Nagata) against HPOmp protein by WB. Lanes S and 1 to 6 correspond to the same numbers as in panel A. Lane L is *N*-lauroylsarcosine-treated HPOmp. Molecular mass markers (M) are shown to the left of columns.

tration was used as a coated antigen (HPOmp ELISA) and the assays were performed with serial dilutions of the test serum. For standardization of antibody titer in the serum, we used a positive control serum obtained from an *H. pylori*-infected patient who was confirmed to have a high antibody titer (by GAP IgG test) (12). The EU value of the HPOmp ELISA was considered to be 300 EU at 1:5,000 dilution. This was based on the results of two series of experiments. In the first experiment, a linear relationship was detected between the reciprocal two-fold dilutions of the positive serum from 1:250 to 1:32,000 and the optical density at 414 nm (OD_{414}). In the second experiment, four other antibody-positive sera showed a similar linear relationship. EU of the test serum was determined from the standard curve of the positive control serum. The second antibody of HPOmp ELISA was used with peroxidase-labeled anti-human immunoglobulin γ -chain F(ab')₂ fragments (American Qualex, La Miranda, Calif.). Absorbance was measured at OD_{414} in the presence of 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (Wako Pure Chemical Industries, Osaka, Japan) as a substrate.

WB was performed as described previously (10). Briefly, the antigen used in ELISA was subjected to SDS-12.5% PAGE and electroblotted onto a polyvinylidene difluoride membrane. The blotted membrane was reacted with 1:5,000-diluted sera and subsequently reacted with 1:5,000-diluted peroxidase-conjugated goat anti-human immunoglobulin γ -chain (Cappel, Malvern, Pa.). The membrane was subjected to an ECL kit-WB detection system (Amersham Japan, Tokyo, Japan) and exposed to X-ray film.

Statistical analysis. Data are expressed as the means \pm standard deviations. Differences between groups were analyzed for statistical significance by the Student's *t* test. All *P* values were two-sided, and values of <0.05 were considered statistically significant.

RESULTS

Presence in *H. pylori*-infected patients of antibodies that reacted with a 29-kDa protein of HPOmp. Membrane proteins were fractionated by SDG separation. The main proteins that floated onto the membrane fraction between 25 and 45% of the SDG (Fig. 1A, lanes 1 to 3) appeared to be large, urease subunit B (UreB), and small, urease subunit A (UreA) subunits as well as heat shock protein 60, based on their molecular sizes. However, these membrane fractions were considered to

be part of the inner membrane consisting of plasma membrane structure (data not shown), since the fractions were completely solubilized with *N*-lauroylsarcosine. On the other hand, a single, opalescent brownish band generated at approximately 55% (wt/wt) of SDG (Fig. 1A, lane 5) was different from the upper fractions in the SDS-PAGE pattern and was resistant to *N*-lauroylsarcosine. SDS-PAGE profiles of this fraction showed relatively clear bands with medium to large molecular sizes and fuzzy bands with small molecular sizes (Fig. 1A, lane 5). The urease activity assay (data not shown) and WB with anti-UreA monoclonal antibody showed that urease did not contaminate this fraction, suggesting that the fraction consisted mainly of HPOmp (Fig. 1C, lane 5).

In the initial step, we examined, using WB, the immunoreactivity of the fraction (HPOmp) against IgG of a patient infected with *H. pylori* (Fig. 1B) and serum samples obtained from *H. pylori*-infected patients (Fig. 2). For each subject, immunoblot analysis showed as the major band a band with a molecular mass of approximately 29 kDa, which was reactive to the serum sample (Fig. 2A). Because the HPOmp fraction could not have been contaminated with UreA, the major band showing a 29-kDa mobility was considered to react with an *H. pylori* protein different from urease.

The results of WB analysis of serum samples obtained from 12 patients before eradication therapy and 6 months after the end of the therapy showed that the intensity of the 29-kDa major band was diminished after treatment compared with that before treatment (Fig. 2A and B). Furthermore, there was a total absence of reactivity in several serum samples obtained at 6 months after treatment. Therefore, we used HPOmp in further studies in order to assess the antibody response in patients

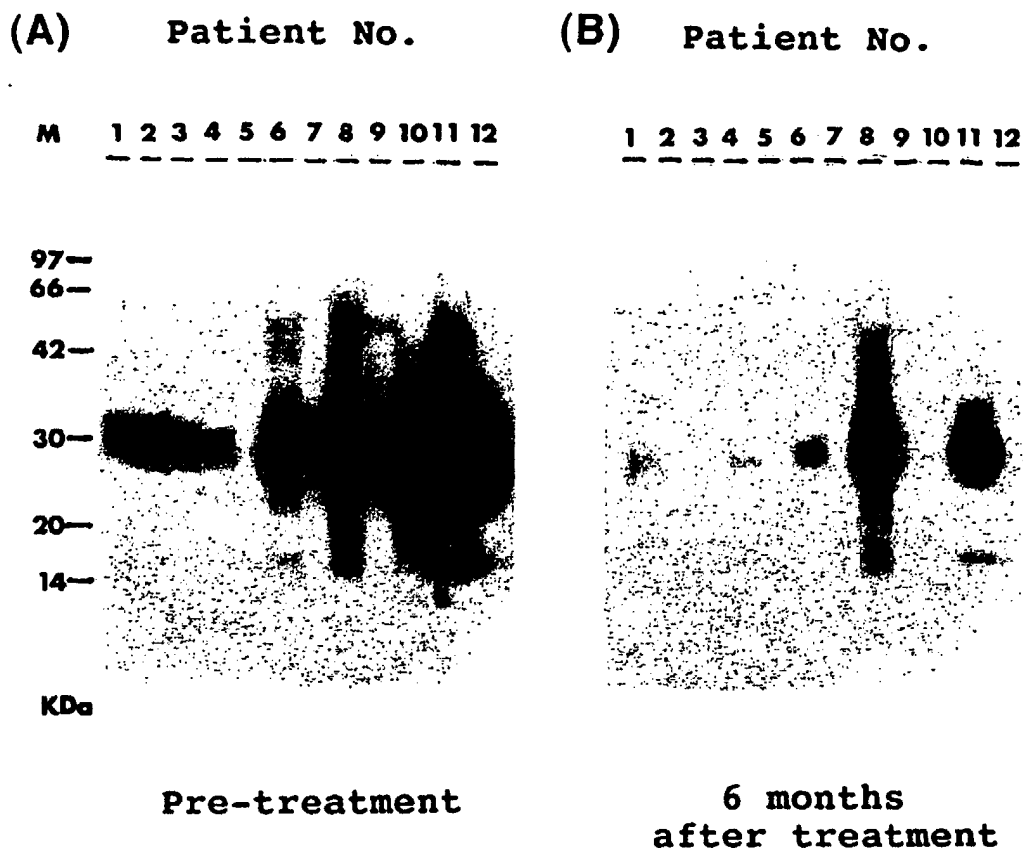


FIG. 2. Immunoreactivities detect by WB analysis at pretreatment and at 6 months after treatment for responders. Fraction 5 shown in Fig. 1A was used for WB as HP omp. Immunoreactivities against HP omp protein of serum samples obtained from 12 patients before treatment (A) and at 6 months after therapy (B) are shown. The molecular mass markers (M) are shown in the left column.

with *H. pylori* infection and to monitor the effectiveness of eradication therapy.

Accuracy of anti-HP omp ELISA. We next assessed the specificity of the newly developed ELISA system with HP omp as

an antigen. Figure 3 shows the distribution of anti-HP omp EU in *H. pylori*-infected patients and the control group. Although a few samples, even from the control group, exhibited somewhat high EU values on HP omp ELISA, the EU for serum

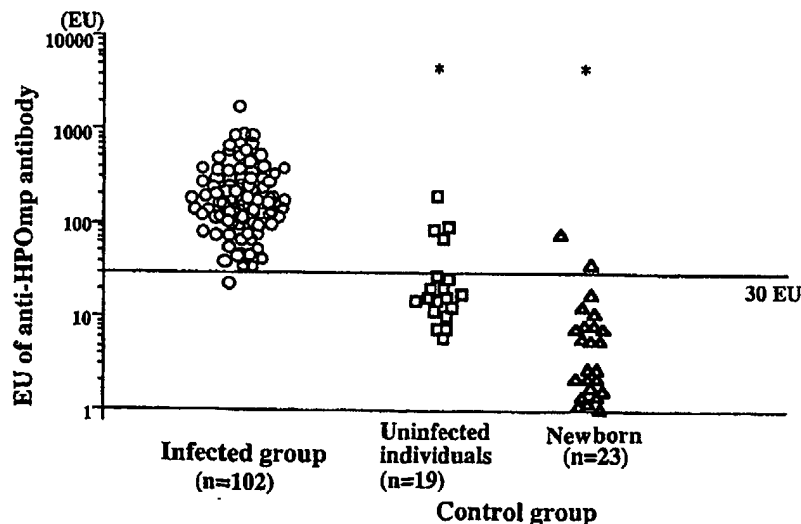


FIG. 3. Distribution of EU for anti-HP omp antibody detected in the sera of *H. pylori*-infected and control individuals. *, $P < 0.005$, compared with the infected group.

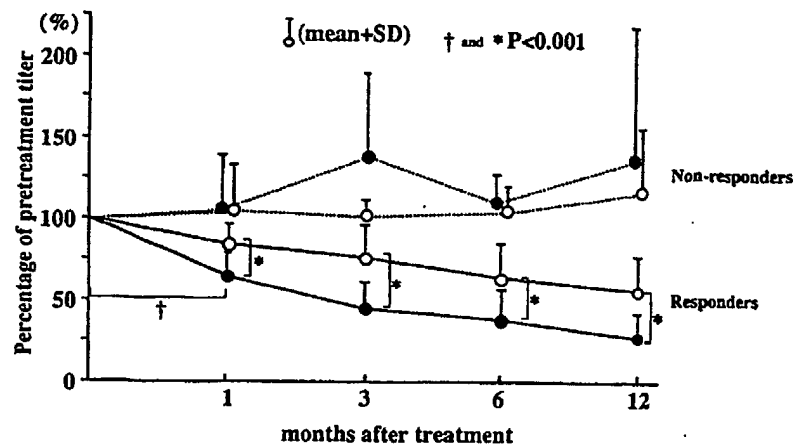


FIG. 4. Serial changes in titers of serum IgG antibody against HPOMP protein for responders and nonresponders. Titers obtained before eradication therapy and at 1, 3, 6, and 12 months after the end of therapy are expressed as percentages of the individual pretreatment titers measured by HPOMP ELISA and GAP IgG ELISA. Data are mean percentages of pretreatment titers \pm standard deviations. Closed circles, titer measured by HPOMP ELISA; open circles, titer measured by GAP IgG ELISA; solid lines, titer in responders; dotted lines, titer in nonresponders. † and *, $P < 0.001$ compared with the pretreatment titer and with the GAP IgG ELISA titer, respectively.

IgG antibody against HPOMP in *H. pylori*-infected patients was clearly higher than that in the control group. It is not clear whether such moderately high titers in the control group arose due to a specific antibody; however, it is possible that antibodies against proteins of the outer membrane of other bacteria may exhibit nonspecific reactions.

When the cutoff was set at 30 EU (Fig. 3), HPOMP ELISA had a sensitivity of 99% (101 of 102 *H. pylori*-infected patients) and specificity of 88% (37 of 42 controls). Therefore, the diagnostic accuracy of HPOMP ELISA was almost equal to that of the commercially available ELISA in evaluating the status of *H. pylori* infection (3, 4). Interestingly, the titer on HPOMP ELISA for pretreatment samples obtained from responders was higher than that for samples obtained from nonresponders after eradication therapy. However, these levels could not be used for the prediction of the effectiveness of therapy prior to this treatment (data not shown).

Changes in *H. pylori*-specific IgG antibody titers after eradication therapy. As shown in Fig. 4, the average titer of anti-HPOMP IgG in responders ($n = 68$) at 1 month after the end of therapy was significantly decreased, by 34.8%, compared with pretreatment levels ($P < 0.001$). In addition, the titer was significantly lower than the average anti-*H. pylori* IgG titer determined by conventional GAP IgG ELISA (16.1% reduction; $P < 0.001$). At 3, 6, and 12 months after eradication therapy, the anti-HPOMP IgG titers were further decreased to 44.9%, 37.5%, and 27.0% of the pretreatment titers, respectively. These levels were also significantly lower than the corresponding GAP IgG titers. On the other hand, in nonresponders ($n = 23$), the average titer of anti-HPOMP IgG at 1 month after treatment was 101.5% of the average pretreatment titer, and during the 12 posttreatment months the titers did not diminish relative to pretreatment levels. Retrospective follow-up studies of responders showed the reappearance of *H. pylori* infection in four patients diagnosed at 6 months after therapy (Fig. 5, responders). In these patients, the reduction in antibody titer was minimal at 1 month after treatment and the titers increased somewhat in two patients. Antibody titers in these patients at 3 months after therapy were significantly higher than those at 1 month after therapy (data not shown). Such cases should be classified as *H. pylori* reinfection or recrudescence.

Usefulness of HPOMP ELISA for the assessment of eradication at 1 month after therapy. When $>25\%$ reduction in anti-HPOMP IgG titer at 1 month after treatment was taken as the cutoff for eradication of *H. pylori* for the test system employed in this study, the sensitivity and specificity of the test were 75% (51 of 68 responders) and 96% (22 of 23 nonresponders) (Fig. 5), respectively. As stated above, patients with recrudescence of *H. pylori* after 6 months showed relatively high titers on HPOMP ELISA at 1 month after treatment, suggesting that amounts of residual organisms nondetectable by bacterial cultures caused a low level of stimulation of the immune system.

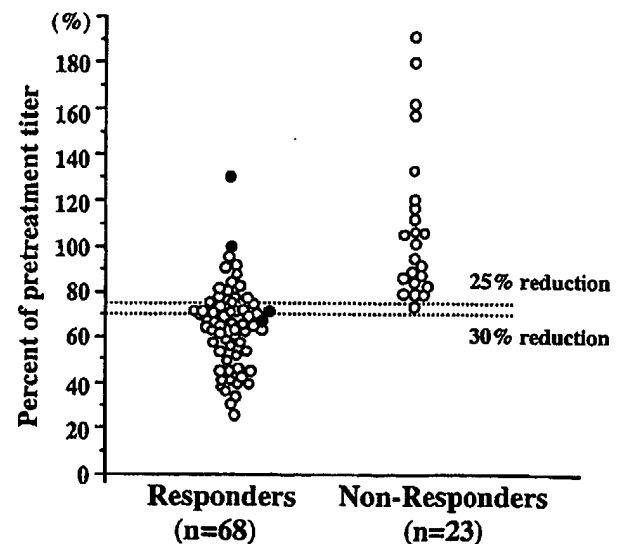


FIG. 5. Specificity and sensitivity of HPOMP ELISA for assessment of response to therapy at 1 month after the end of therapy. Dotted lines indicate the relative percentages corresponding to 25 and 30% reductions compared to the pretreatment titer. Closed circles, percentages of pretreatment titers for HPOMP ELISA for four individuals who were categorized as responders but showed evidence of reappearance of *H. pylori* infection at 6 months after therapy.

DISCUSSION

Eradication of *H. pylori* markedly improves the natural history of peptic ulcer in patients with duodenal or gastric ulcers (5). Confirmation of eradication of the causative organism usually requires noninvasive and invasive tests involving repeated endoscopic examinations. UBT and *H. pylori* antibody assay are widely used as noninvasive tests for detection of *H. pylori*. Since the results of UBT reflect the presence of *H. pylori* in the whole stomach, sampling error in collection of biopsy specimens very seldom occurs, and therefore, UBT is a clinically useful diagnostic test with a high sensitivity and specificity. However, the ^{13}C -UBT requires a mass spectrometer, which is expensive and is not readily available to general practitioners (6). On the other hand, there are several problems with ^{14}C -UBT such as protection against radiation pollution caused by the use of radioactive material and its unsuitability for infants and pregnant women (1). The serological tests are less expensive and easier to perform than UBT and may be preferable for the diagnosis of *H. pylori* infection. Therefore, serological diagnosis has been used in seroepidemiological studies and screening of *H. pylori* infection in large samples (3).

H. pylori causes a chronic infection of the gastric mucosa, and humoral and cellular immune responses persist for a long period unless the organisms are eradicated (9). The currently available *H. pylori*-specific antibody assay has not been used for the early monitoring of eradication, since the antibody titer gradually decreases after disappearance of the organism. Thus, it is important to establish an improved *H. pylori*-specific antibody assay for use as a noninvasive test for the early and accurate assessment of the response to treatment. In this study, we demonstrated that the 29-kDa outer membrane protein of *H. pylori* (HPOMP) is a major antigen capable of inducing a strong antibody response and that a new ELISA system incorporating the HPOMP is capable of detecting the eradication of *H. pylori* with high sensitivity and specificity compared to the serological tests so far established.

Several investigators reported that the titer of *H. pylori*-specific antibody decreases progressively in responders after treatment (2, 7, 8). The average time necessary for 50% reduction in antibody titer varies from one study to another. For example, Kosunen et al. (8) and Cullen et al. (2) reported that the average duration until antibody titers fell by 50% in response to treatment was 3 or 4 months. On the other hand, Hirschl and coworkers (7) reported that a 50% reduction in IgG titer was observed more than 6 weeks after treatment. The differences in the intervals between cessation of therapy and reduction in the antibody titer might be due to the different antigens used in the ELISAs: an acid-glycine *H. pylori*-extract was employed by the former groups of investigators and a 120-kDa protein of *H. pylori* was used by the latter investigators. The present study using HPOMP detected 34.8% and 55.1% reductions in antibody titers relative to the pretreatment level at 1 and 3 months after therapy, respectively.

The kinetics of posttreatment changes in antibody titer have shown some reduction during the early posttreatment period irrespective of the success of bacterial eradication (8). In our study, a few nonresponders also showed a decrease in the antibody titer (Fig. 5). Therefore, it is difficult to determine the success or failure of treatment in the early posttreatment period (8).

The major finding of the present study was the reduction in the intensity of the bands in the low-molecular-mass region detected 6 months after therapy by WB analysis with the outer membrane protein as the antigen. This feature indicates that HPOMP is a useful diagnostic indicator reflecting the efficacy

of eradication therapy. Our new ELISA with HPOMP as an antigen was capable of detecting a significant reduction in the titer of *H. pylori*-specific antibody at 1 month after treatment with high sensitivity and specificity (75% and 96%, respectively; Fig. 5). The reason for the rapid reduction in the titer of HPOMP ELISA in responders compared with conventional ELISA is unknown at present. It is possible, however, that the mode of colonization of the body by *H. pylori* is limited and only superficial in the gastric mucosa and that the clearance of organisms caused by eradication therapy might induce a rapid decline in specific immune responses against the organism. It is also possible that HPOMP might be one of the immunodominant antigens of this organism reflecting infection with *H. pylori*, since the HPOMP is probably located on the bacterial surface, while the antigens used in conventional ELISA consist of the whole antigenic material of the organism including cytoplasmic components.

We also evaluated the sensitivity and specificity of HPOMP ELISA in the early assessment of the efficacy of eradication therapy. Our results showed that the sensitivity and specificity of the novel tests are similar to those of UBT. Previous reports showed that the sensitivity and specificity of the UBT for eradication of infection at 6 weeks after treatment were 97 to 99% and 71 to 76%, respectively (13, 14). The sensitivity of the UBT is superior to that of serological tests. On the other hand, if the cutoff value of reduction in antibody titer as the criterion for bacterial eradication is set at >30%, the specificity of assessment of eradication by HPOMP ELISA is 100%, suggesting that our new HPOMP ELISA can accurately evaluate the success of bacterial eradication at an early stage and that the test is more specific than the UBT.

Finally, our results showed that a protein with a molecular mass of approximately 29 kDa induces a strong antibody response. We tentatively named the highly immunogenic protein HPOMP29. Our amino-terminal sequencing of the protein showed that HPOMP29 does not exhibit any identity to previously reported proteins (data not shown). However, according to comparison with the complete genome nucleotide sequence of *H. pylori* 26695 that was reported recently (15), HPOMP29 is categorized as a member of a family of outer membrane proteins of *H. pylori*. Further studies are necessary to characterize the structure and function of this newly isolated outer membrane protein.

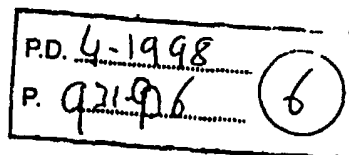
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Use of Immunoblot Assay To Define Serum Antibody Patterns Associated with *Helicobacter pylori* Infection and with *H. pylori*-Related Ulcers

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Serology has been used worldwide to detect *Helicobacter pylori* infection. Using an immunoblot assay with an antigen from strain ATCC 43579, we sought to determine the antibodies which were good markers of colonization and the antibody patterns associated with ulcers or atrophy. Out of 98 dyspeptic patients, 41 were colonized by *H. pylori*, based on a positive culture or on positive results of both a urease test and direct examination. These 41 patients were seropositive by an enzyme immunoassay, and 12 of them had ulcers and 29 had evidence of atrophy. Fifty-seven of the 98 patients were noncolonized. Twenty-five of the 57 had evidence of gastric atrophy, and 10 were seropositive; 5 of these 10 had ulcers. By Western blot analysis, 12 antibodies were significantly more frequent in sera from colonized patients, and they produced immunoreactive bands at 125, 87, 74, 66, 54, 48, 46, 42, 35, 30, 16 and 14 kDa. The presence of at least one band at 54, 35, or 42 kDa was the best marker of infection (sensitivity, 95%; specificity, 82%). In the group of colonized patients, none of the antibody patterns were correlated to gastric atrophy. Conversely, the presence of a band at 125, 87, or 35 kDa was statistically associated with the presence of an ulcer. The simultaneous presence of bands at 87 and 35 kDa predicted the risk of ulcers with 83% sensitivity and 69% specificity. By using CagA-positive and VacA-positive strains and CagA-negative and VacA-negative isogenic mutants, the antigens corresponding to the bands at 125 and 87 kDa were shown to be CagA and VacA, respectively. On the other hand, the 35-kDa antigen is a novel uncharacterized component of *H. pylori*. These results may help to optimize the composition of antigenic preparations for serologic detection of *H. pylori* colonization. Immunoblot assay would be useful for screening patients at high risk of ulcers.

Helicobacter pylori is an important etiologic factor for chronic gastritis and peptic ulcers. It is also associated with gastric atrophy, which can lead to adenocarcinoma, and with gastric lymphoma (3-5, 17, 18, 23, 24, 29, 31, 39). The diagnosis of *H. pylori* gastric infection can be conducted by using direct (invasive) or indirect (noninvasive) methods (28). Among the indirect methods, serology is a valuable tool for seroepidemiological studies (43, 45) or for posttreatment follow-up (46). The serological assays are, essentially, enzyme immunoassays (EIAs) with a variety of antigenic preparations. The performances of the EIAs are hampered by cross-reactions (33) and there is no consensus as to the best antigenic preparation to use for *H. pylori* serology (47). Consequently, it would be of interest to know which antigens of *H. pylori* should be included in an ideal preparation designed for serodiagnosis of *H. pylori* infection.

Although all *H. pylori*-infected subjects have gastritis, a considerable number remain asymptomatic whereas others develop severe diseases, such as ulcers, gastric lymphomas, atrophic gastritis, or adenocarcinomas (9). It would be valuable to have predictive markers for severe diseases at our disposal. However, the factors influencing the evolution of *H. pylori* gastritis remain poorly understood; they could be related to the infecting strain or to the host response (5, 31). The ability of certain strains to produce a vacuolating cytotoxin encoded by the *vacA* gene has been associated with more severe illnesses

(26, 40, 44). Most, but not all, of the cytotoxic strains express the CagA antigen, which has been associated with a more severe inflammatory response (4). The vacuolating toxin and the CagA antigen elicit specific antibodies during infection (4, 6, 7, 32, 49), but the value of these antibodies as predictive factors for the severity of the disease remains controversial (10, 23, 24, 44).

Because it depends on both the characteristics of the strain and the host response, the serum antibody response to *H. pylori* could provide clues in predicting the severity of *H. pylori*-associated diseases. Several studies have demonstrated a strong correlation between the levels of total anti-*H. pylori* immunoglobulin G (IgG) and the colonization of the gastric mucosa by the bacteria (1, 27, 37). However, the anti-*H. pylori* antibody patterns have been reported to show a high degree of polymorphism (2, 16, 30, 33). This antibody polymorphism could be related to the pathological status and thus may serve as a biological predictor of the type of disease associated with the *H. pylori* infection. In 1993, Xiang and colleagues described an EIA with a recombinant antigen including a fragment of the CagA protein (48). They demonstrated a positive correlation between the EIA and the Western blotting methods used to detect the anti-CagA antibodies. There was also a strong correlation between the anti-CagA antibody level and the presence of an ulcer. Nevertheless, other antibodies or combinations of antibodies may also be good markers of the severity of the disease.

In this work, we studied the frequencies of the antibodies to 12 major antigens of *H. pylori* in the sera of 98 patients clinically and histologically documented. We sought to determine the antibodies which are the best markers of colonization and

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the antibody patterns associated with the presence of an ulcer or a gastric atrophy.

MATERIALS AND METHODS

Patients. A total of 98 consecutive patients (34 males and 44 females) examined in the Hepato-Gastro-Enterology Department of the University Hospital Center of Poitiers, France, were included in the study between 1995 and 1996. The median ages were 51.4 years (range, 12 to 85 years) and 44.3 years (range, 15 to 79 years) for males and females, respectively. The patients presented with dyspeptic syndrome and underwent an upper gastrointestinal endoscopy with multiple antral and fundic biopsies. They had received neither antimicrobial nor antacid therapies during the previous 3 months. The biopsies were processed for culture of *H. pylori* and for histology. Sera were collected the day of the endoscopy; they were aliquoted and frozen at -80°C until they were used.

Bacteriology. Gastric biopsy specimens were placed into sterile 0.15 M NaCl solution and transported to the laboratory within 30 min. A part of each specimen was ground and inoculated into a nonselective Columbia blood agar (bioMérieux, Marcy l'Etoile, France). The plates were incubated at 37°C under microaerobic conditions for 10 days. The isolates were identified as *H. pylori* by Gram staining and urease, oxidase, and catalase activities. A part of the ground specimen was smeared and Gram stained for direct search for spiral bacteria. A second part of each specimen was placed into 0.2 ml of 20 mM urea, containing phenol red as a pH indicator, for detection of urease activity. Urease reactions were recorded after 1 h of incubation at 37°C .

Histology. Gastric specimens were placed into 10% formalin, and multiple sections of each specimen were hematoxylin-eosin or Giemsa stained. Chronic and active chronic gastritis scores were assigned to each biopsy specimen, and these scores were used for classifying the patients into the following categories: (i) normal, (ii) with gastritis, and (iii) with atrophic gastritis. A gastritis score of 0 indicated that no mononuclear cells were present, a score of 1 indicated that mononuclear cells were present in a patchy distribution, a score of 3 indicated a very dense infiltration of mononuclear cells throughout the entire section, and a score of 2 was intermediate between 1 and 3. Similar criteria for polymorphonuclear leukocytes were used for grading acute inflammation (36). In this work, the patients with a gastritis score of ≥ 1 were considered gastritis patients. Acute and nonacute gastritis were classified into the same group. The gastric atrophy was scored from 1 to 4 on the basis of the degree of atrophy of the glands and the density of mucus-secreting cells. The patients with a score of ≥ 1 were considered to have gastric atrophy. The presence of spiral bacteria was noted on the Giemsa-stained smear. The presence of an ulcer was noted during the endoscopic examination. Histological, bacteriological, and serological statuses of the patients were established blindly by three independent investigators.

***H. pylori* strains and antigenic extracts.** Hydrophilic antigens from *H. pylori* ATCC 43579 were extracted by a method previously described (2, 13). The strain was CagA positive and produced a VacA vacuolating toxin. *H. pylori* was cultured on chocolate agar plates and incubated at 37°C under 5% O_2 for 48 h. Bacterial cells from each plate were harvested and suspended in 2 ml of sterile 0.15 M NaCl at 4°C . The bacterial suspension was gently vortexed for 60 s, and then the cells were sedimented by centrifugation (10,000 $\times g$ for 20 min at 4°C). The supernatant was dialyzed overnight at 4°C against 0.15 M NaCl; the protein concentration of the resulting saline extract was determined by the bicinchoninic acid method (Pierce Chemicals, Rockford, Ill.), and the extract was frozen at -80°C until it was used in immunoassays.

Whole-cell extracts were prepared from the *H. pylori* wild-type strain 84-183 (ATCC 53726) and from the CagA-negative isogenic mutant 84-183:M21 (42). These extracts were obtained by the lysis of bacterial cells cultured for 48 h on chocolate agar at 37°C under 5% O_2 . Bacterial cells from each plate were harvested and suspended in 2 ml of sterile 0.15 M NaCl at 4°C . The cells were broken by ultrasonic treatment in a Sonifier 450 (Bransson, Oss, Paris, France). After centrifugation at 15,000 $\times g$ for 15 min, the supernatant was dialyzed against 0.15 M NaCl for 48 h.

Vacuolating toxin (VacA)-enriched preparations were obtained as previously described (8). Briefly, the *H. pylori* 60190 (ATCC 49503) wild-type strain and 60190-v1, a mutant strain that does not produce VacA, were cultured for 3 days at 37°C under 5% O_2 in tryptic broth supplemented with 10% fetal bovine serum and 5% β -cyclodextrin (34). The bacterial cells were pelleted by centrifugation at 2,000 $\times g$ for 10 min, and the supernatant was dialyzed against 0.15 M NaCl for 48 h and frozen at -80°C until use.

The strains *H. pylori* 60190 (ATCC 49503), 60190-v1, 84-183 (ATCC 53726) and 84-183:M21 were kindly donated by T. Cover, Vanderbilt University, Nashville, Tenn.

Determination of antibody levels by ELISA. To assess IgG antibodies to *H. pylori* in the human sera, we used an enzyme-linked immunosorbent assay (ELISA) with saline extract from strain ATCC 43579 as the antigen (14). Briefly, 96-well microtiter plates were coated with 250 ng of antigenic protein per well, and the sera were diluted 1:100. The assay was calibrated by using a reference serum included on each plate. The serum specimen and the reference serum were assayed in triplicate. For each serum, results were expressed as an ELISA index obtained by calculation of the ratio of the mean optical density of the serum specimen to the mean optical density of the reference serum. The cutoff

value was determined by the construction of a receiver operating characteristic curve (14).

Immunoblot assays. Using a Maxi-Gel apparatus (Bio-Rad, Richmond, Calif.), we carried out sodium dodecyl sulfate-polyacrylamide gel electrophoresis of bacterial extracts as described by Laemmli (25), with a 4% stacking gel and a linear gradient (8 to 16% acrylamide) separating gel (16 by 18 cm). Prior to electrophoresis the antigenic extracts were heated at 100°C for 5 min in Tris-HCl buffer (pH 6.8) containing 1% sodium dodecyl sulfate and 10% β -mercaptoethanol. Preparative slab gels were loaded with samples containing 1 mg of protein. A sample of molecular mass markers (Bio-Rad) was loaded on each gel. Migrations were performed under a constant current of 35 mA until the bromophenol blue dye migrated out of the gel. The proteins were then transferred for 1 h onto prewetted nitrocellulose membranes (Bio-Rad) by using an electrophoretic transfer cell (Trans-Blot; Bio-Rad) under a constant current of 200 V. Following the protein transfer, the nitrocellulose sheets were cut into strips. The strip corresponding to the molecular mass markers was stained with Ponceau red (Sigma) and kept for calibration purposes. The blot strips were incubated for 1 h with the patients' sera diluted 1:250 in with calibrating monospecific sera (see below) appropriately diluted. The strips were then rinsed three times in Tris-saline blotting buffer (pH 8) and incubated for 3 h in alkaline phosphatase-conjugated anti-human IgG (Dakopatts, Copenhagen, Denmark). After being washed, they were developed with 5-bromo-4-chloro-3-indolylphosphate as the substrate and with nitroblue tetrazolium as the chromogenic indicator. The reactions were stopped after 15 min by washing the strips thoroughly with distilled water. In order to normalize the positions of the immunoreactive bands detected by the patients' sera we used, as internal references, five monospecific polyclonal rabbit sera raised against the following five high-performance liquid chromatography-purified antigens of *H. pylori*: (i) an antigen of 26 kDa purified from *H. pylori* ATCC 43579, (ii) UreA (30 kDa) purified from *H. pylori* ATCC 43579, (iii) UreB (66 kDa) purified from *H. pylori* ATCC 43579, (iv) the vacuolating toxin (87 kDa) purified from *H. pylori* 60190 (ATCC 49503) (7), and (v) the CagA antigen (125 kDa) purified from *H. pylori* 84-183 (42). The first three sera were generously supplied by Patrice Méhennecy Cunnough (Marcy l'Etoile, France), and the others were kindly donated by T. Cover. When tested by Western blotting with a saline extract from *H. pylori* ATCC 43579, these calibrating sera reacted with antigens having the expected apparent molecular masses (Fig. 1, lanes 1 to 5). The positions of the immunoreactive bands revealed by the patients' sera were assessed with a calibrating curve constructed by plotting the distances of migration (in millimeters) of the immunoreactive bands obtained with the calibrating sera against the molecular masses (in kilodaltons) of the corresponding antigens. The polynomial regression showed a R^2 coefficient of 0.995. The calibrating sera and the patients' sera were studied simultaneously in the same gels. To assess the reproducibility of the migration distances, experiments were carried out four times with the monospecific sera. The coefficients of variation (i.e., the ratio of standard deviations (SD) to means) of the migration distances were $<10\%$.

RESULTS

Status of patients. Colonized patients were defined as patients who were positive by culture of *H. pylori* or patients who were positive for spiral bacteria by direct examination of biopsy specimens and who also had positive urease tests. Noncolonized patients were defined as patients who were negative by culture, direct examination, and urease tests. The serum antibody levels, expressed as the ELISA index (see Materials and Methods), ranged from 0.02 to 1.08 for the 98 patients. To define a patient's serologic status, a cutoff value of 0.15 was determined by the receiver operating characteristic curve method (14), using the direct methods of diagnosis (i.e., culture, direct examination, and urease test) as a "gold standard." Under these conditions, the EIA showed a sensitivity of 100% and a specificity of 83% for predicting *H. pylori* colonization.

Of the 98 patients, 41 (41.8%) were colonized by *H. pylori* and were seropositive. Among these 41, 12 had ulcers and 29 had evidence of gastric atrophy (i.e., scores from 1 to 3; median = 1.8). Fifty-seven (58.2%) of the 98 patients were noncolonized. Among the 57, 25 had evidence of gastric atrophy (i.e., scores from 1 to 3; median = 1.6) and 10 were seropositive (5 of these had ulcers). Colonized and noncolonized patients were similar in age (mean = 45.9 years [SD = 18.3 years] and mean = 48.5 years [SD = 21.4 years], respectively).

Antibody patterns. Sera from seropositive patients tested by Western blotting revealed from 1 to 15 bands (average 8.1 bands), while sera from seronegative patients revealed from 0

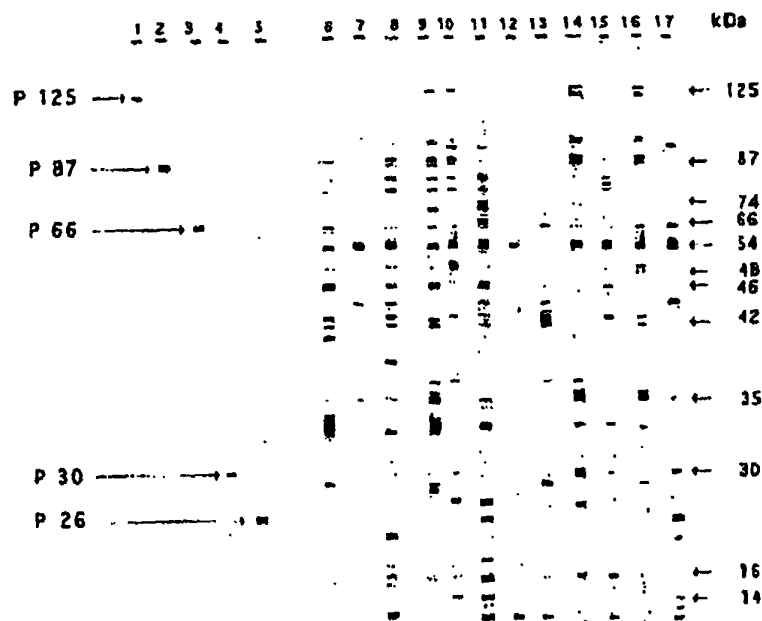


FIG. 1. Immunoblot patterns obtained with a saline extract from *H. pylori* ATCC 43579 with five rabbit sera raised against purified antigens from *H. pylori* (lanes 1 to 5) and with 12 selected sera from patients infected with *H. pylori* (lanes 6 to 17). Molecular masses are indicated on the right. The arrows indicate immunoreactive bands corresponding to the antigens p125, p87, p66, p30, and p26. The figure shows a scan of the original nitrocellulose strips.

to 5 bands (average, 1.4 bands). A set of blots with sera from seropositive patients and with calibrating sera is depicted in Fig. 1.

The blots were analyzed by three independent investigators. Seventeen different bands were distinguished on the 98 blots. Of the 17, four bands (62, 25, 23, and 13 kDa) were disregarded because their frequencies were not significantly different in the colonized and the noncolonized patients (chi-square test; $P > 0.05$). An additional band at 19 kDa was disregarded because its frequency was low. The abilities of the 12 remaining immunoreactive bands to predict colonization were evaluated by comparing the frequency of each band in the 41 colonized patients with that in the 57 noncolonized patients (Table 1). The best performance indexes were obtained with bands at 54, 42, and 35 kDa (the performance index is the percentage of patients correctly classified as colonized or noncolonized by the presence or the absence of the specific band). The presence of at least one of these three bands predicts colonization with 95% sensitivity and 82% specificity. These performances could not be improved by considering the presence of any other bands.

Correlations between antibody patterns and pathological status of colonized patients. We next focused on the 41 colonized patients to see whether the presence of ulcers or atrophy could be correlated with the presence of certain antibody patterns. We compared the frequencies of the 12 antibodies in the populations of patients showing and not showing evidence of ulcers by endoscopy or evidence of gastric atrophy by histology. We failed to demonstrate significant relationships between the presence of gastric atrophy and the antibody pattern. Conversely, a significant relationship (chi-square test; $P \leq 0.05$) was established between the presence of a band at 125, 87, or 35 kDa and the presence of ulcers (Table 2). Next we tested

different combinations of antibodies for their abilities to predict the presence of ulcers. The best performance index was obtained by the combination of antibodies to both the 87- and the 35-kDa antigens. The simultaneous presence of these two antibodies predicted a risk of ulcer with 83% sensitivity and 69% specificity.

Partial characterization of p125, p87, and p35. To demonstrate that antigens corresponding to the immunoreactive bands at 125 (p125) and 87 kDa (p87) were actually CagA and

TABLE 1. Frequencies of 12 antibodies to *H. pylori* in 98 human sera and their abilities to predict *H. pylori* infection

Immunoreactive band (kDa)	No. (%) of reacting sera from:		Performance index (%) ^b
	Colonized patients (n = 41) ^a	Noncolonized patients (n = 57) ^a	
125	29 (70.7)	11 (19.3)	77
87	25 (60.9)	11 (19.3)	72
74	14 (34.1)	6 (10.5)	66
66	19 (46.3)	9 (15.8)	68
54	34 (82.9)	10 (17.5)	83
48	14 (34.1)	4 (7.0)	68
46	22 (53.7)	8 (14.0)	72
42	24 (58.5)	4 (7.0)	79
35	26 (63.4)	6 (10.5)	79
30	26 (63.4)	17 (29.8)	67
16	20 (48.8)	1 (1.8)	78
14	15 (36.6)	7 (12.3)	66

^a Based on urease assay, direct examination, or culture. The antibodies to the 12 antigens used were significantly more frequent in the patients infected by *H. pylori* than in the noninfected patients (chi-square test; $P < 0.05$).

^b Percentage of patients correctly classified [i.e., (number of true positive + number of true negative)/number of patients].

TABLE 2. Frequencies of three antibodies to *H. pylori* in 41 sera from *H. pylori*-colonized patients,* with or without gastric ulcers,† and abilities of the antibodies to predict ulcers

Immunoreactive band (kDa)	Prevalence (%) of antibodies in patients†		Performance Index (%)‡
	Ulcer (n = 12)	No ulcer (n = 29)	
125	10 (83.3)	18 (62.1)	51
87	10 (83.3)	14 (48.3)	61
35	12 (100.0)	14 (48.3)	66

* Based on urease assay, direct examination, or culture.

† Based on endoscopy.

‡ The antibodies to the three antigens listed were significantly more frequent in patients with ulcers than in nonulcerous patients (chi-square test; $P \leq 0.05$).
Percentage of patients correctly classified [i.e., (number of true positive + number of true negative)/number of patients].

VacA, respectively, we prepared bacterial extracts with strains known to express these antigens along with extracts from isogenic mutants which no longer express the specified antigens. We carried out immunoblot assays with eight selected sera showing immunoreactive bands at 125 kDa and eight sera showing immunoreactive bands at 87 kDa. All the sera selected for having antibodies to p125 showed an immunoreactive band at 125 kDa with the extract from the CagA-positive strain 84-183, as did the anti-CagA rabbit serum. Conversely, the 125-kDa band was absent when these sera were tested against an extract from the CagA-negative isogenic mutant 84-183: M21. Similarly, we used a VacA-enriched preparation from the VacA-positive strain 60190 and from the VacA-negative isogenic mutant 60190::v1 to test eight sera found to have antibodies to p87. All these sera and the anti-VacA control serum showed immunoreactive bands at 85 to 87 kDa when tested with the antigenic preparation from the VacA-positive strain, whereas this band was absent when an extract from the VacA-negative mutant was used as the antigen (Fig. 2). These results suggest that p125 and p87 represent the CagA and the VacA antigen, respectively. Because VacA is known to include a 37-kDa subunit (24, 35), we hypothesized that the 35-kDa antigen (p35) represents this subunit. As shown in Fig. 2, the anti-VacA control serum does not react in the 30- to 37-kDa area (lanes C). Moreover, only one of the eight sera tested exhibited an immunoreactive band at 35 kDa when tested against the extract from the VacA-positive strain. This band remained (although slightly shifted) when the extract was pre-

pared from the VacA-negative mutant (Fig. 2, lanes 3+ and 3-). Furthermore, of the 51 seropositive patients, 9 had antibodies to p87 but no antibody to p35 whereas 8 had antibodies to p35 but no anti-p87. Thus, 17 patients (33.3%) had antibodies to only one of the two antigens. These results suggest that p35 is different from VacA and that it is a novel uncharacterized antigen.

DISCUSSION

The choice of a single method for the diagnosis of *H. pylori* infection remains controversial, and at present, two different methods are needed to determine whether a patient is infected (28). The bacteriological method (culture) is unquestionably the most specific, but it is subject to sampling errors and is not very sensitive, giving false-negative results (28). The so-called "global methods" (i.e., breath test and serology) are more sensitive, but they may be less specific. Serology is now widely used as a global method of diagnosis, and a variety of commercially available kits (27) or homemade methods (14) have been described. Most of these methods provide satisfactory results (1, 15, 20, 27, 37, 43); however, significant improvements must be made before they can be considered as reference methods. Improvement of the antigenic preparations is the best way to improve the performances of the serological methods, but to date, there is no consensus as to the most appropriate composition of the preparations. Our data provide indications of antigens that must be included in an "ideal" antigenic preparation for *H. pylori* serology. The immunoblot assay we used was performed under very stringent conditions, and it was normalized to optimize its reproducibility. We used a mixture of components from a single strain as the antigenic preparation. This kind of preparation must contain the major antigens of *H. pylori*, particularly the CagA antigen, the vacuolating toxin (6, 7), the heat shock proteins, the urease complex (11, 12), and certain adhesins (13). The use of an antigenic mixture instead of single purified antigens is consistent with a previous study where we demonstrated that a preparation of extracted antigens is more efficient for serology than single purified recombinant antigens (47). Among the most relevant antigenic fractions, we demonstrated that p125 is the CagA antigen and p87 is the VacA antigen. p54 should be HspB, while p35 and p42 have not yet been characterized. The discrepancies between the results of serology and the results of the direct methods may be due to the lack of sensitivity of the direct methods. The 10 patients who were seropositive and

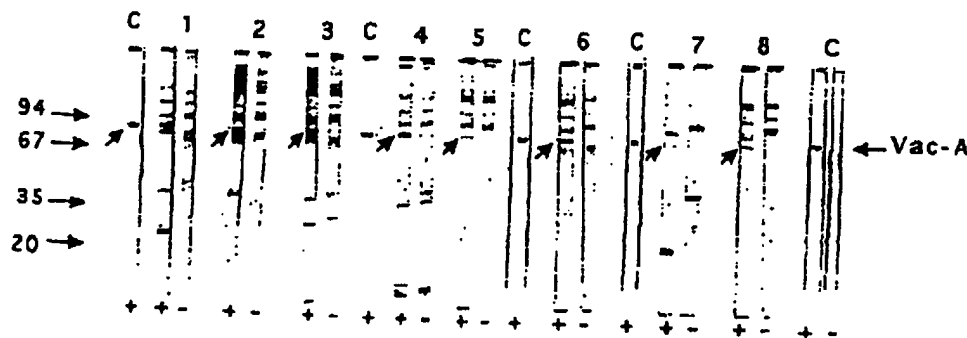


FIG. 2. Immunoblot patterns obtained with vacuolating toxin-enriched preparations from the VacA-positive strain 60190 (+) and the isogenic VacA-negative mutant 60190::v1 (-) with eight selected patients' sera which reacted with p87 from *H. pylori* ATCC 43579 (lanes 1 to 8). Lanes C, serum from a rabbit immunized in nitrocellulose strips.

noncolonized, on the basis of the direct method, might have been falsely classified as noncolonized. Actually, five of them had ulcers and all of their sera gave more than five bands when tested by Western blotting. Moreover, it should be noted that no patients were seronegative and colonized by the direct methods.

Some, but not all, of these data are in agreement with previous work (16, 19, 33). Nilsson et al. found strong correlations between *H. pylori* infection and the presence of antibodies to 110- to 120 (presumably CagA), 26-, 29-, 30-, 31-, and 33 (presumably our p35)-kDa antigens (33). Faulde et al. found four antigens of 130, 93, 75, and 67 kDa to be the most immunogenic during *H. pylori* infection (16). Thus, the correlations among the immunoblot analysis findings of different authors are poor. This fact may be due to both the diversity of the technical conditions and the use of different strains as the sources of antigens. It must be emphasized that the use of immunoblotting as a diagnostic tool implies a good standardization of the method. A well-defined strain representative of the clinical strains should be used to obtain the antigenic preparation, and the assay must be calibrated with well-defined antisera. In an unpublished study, we found that a population of clinically isolated strains could be clustered into three groups according to their antigenic profiles. The major group included the strain ATCC 43579 used in the present work. The strains in this group exhibited a richer antigenic composition than those in the other groups, including antigens of 120 to 125 kDa, 80 to 90 kDa, and 54, 42, and 35 kDa (25a). Thus, the strain chosen in this work as the source of antigens appears to be representative of the strains isolated most frequently in our patients. This strain is commercially available.

H. pylori infection can lead to a variety of diseases. Presently, the only reliable way to identify the illness associated with a *H. pylori* infection remains an endoscopic examination coupled with histologic examination of the gastric mucosa. Attempts have been made before to correlate the severity of the *H. pylori*-associated diseases to the antibody level, the specificity of the serum antibodies, or the isotypes of these antibodies (4, 6, 10, 17, 21, 22). The polymorphism of the antibody response to *H. pylori* has been suspected to reflect either an evolution of the immune response or an antigenic shift of the infecting strain (2, 30). Nevertheless, it has also been suspected to be correlated to a predisposition for severe diseases. The presence of anti-CagA has been associated with the presence of ulcers; however, the relevance of this correlation remains controversial (10, 23, 24, 41, 48, 49). In any case, other serum antibodies may be better markers for predicting severe diseases. In the present work, we found that three single antigens (CagA, VacA, and p35) elicited antibodies more frequently in patients suffering from ulcers. The anti-VacA antibody is a more powerful marker of ulcers than anti-CagA. This is not surprising, because the vacuolating toxin has been suspected to be involved in the mechanism of ulcerous lesions of the mucosa (35, 38, 40, 41, 44). The anti-p35 antibody appears to be the best marker of ulcers, and the simultaneous presence of anti-VacA and anti-p35 antibodies predicts, with good sensitivity, a predisposition to ulcers. The poor specificity observed could be due to the fact that peptic ulcers may be intermittently present and certain patients may be nonulcerous at the time of endoscopic examination but may later evolve to an ulcerous state. On the other hand, none of the serologic markers tested have been able to predict atrophic gastritis. It may be necessary to look for other antibodies, or the atrophy could be unrelated to serologic status. Other authors have attempted to establish correlations between certain antibody patterns and gastric cancer (21).

In conclusion, the antigenic preparations designed for *H. pylori* serology must include CagA, VacA, HspB, and also the uncharacterized antigens p42 and p35. Immunoblot assay would be useful for screening patients at high risk of ulcers. The follow-up of a cohort of *H. pylori*-infected patients may be of interest to confirm the value of these findings.

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Isolation and Preliminary Evaluation of a Low-Molecular-Mass Antigen Preparation for Improved Detection of *Helicobacter pylori* Immunoglobulin G Antibodies

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Previously, immunoglobulin G (IgG) antibodies to five antigens with a relative molecular mass of between 15 and 30 kDa from *Helicobacter pylori* were found to be significantly more frequent in *H. pylori*-infected patients than in noninfected patients. In this study, these specific low-molecular-mass (LMW) antigens were separated by ultrafiltration of whole-cell sonicates. The LMW antigen preparation was evaluated by enzyme-linked immunosorbent assay with serum samples from 76 children with abdominal symptoms and 151 adults. Antibody levels to *H. pylori* were found in serum from 35 (46%) children and 83 (55%) adults. Increased sensitivity, specificity, and predictive value of positive and negative results of the test were higher with LMW antigens than with the heat-stable antigen previously described. The low specificity and predictive value of a positive result were due to seropositive results for 21 persons with a negative culture for *H. pylori* and negative microscopy results for *Helicobacter*-like organisms in biopsies from gastric mucosa. Histologically, chronic gastritis was demonstrated in 43% of these persons, and 19% had peptic ulcer, indicating that they have or have had *H. pylori* infection. Specific antibodies to *H. pylori* were confirmed in all 21 patients by the Western immunoblot technique. Use of the LMW antigen improved the IgG antibody detection in patients with *H. pylori* infection, even though the results reflect the difficulties in establishing a true gold standard for diagnosis of *H. pylori* infection.

Helicobacter pylori has been established as the major cause of chronic gastritis and peptic ulcer (2, 18) and may play an important role in the pathogenesis of gastric cancer (9, 17). Patients with *H. pylori* infection do not always have specific symptoms or specific endoscopic findings which establish the diagnosis (1, 26). The laboratory findings are therefore important for a reliable diagnosis of *H. pylori* infections. One of the cheapest and simplest tests is the detection of antibodies to *H. pylori*. Detection of antibodies may, in contrast to most other tests, also reveal "hidden" infections caused by coccoidal forms (15) or intracellular survival of *H. pylori* in phagocytes (4), which may obscure the diagnosis, especially after treatment. Serological studies by different techniques and with different antigen preparations have shown that patients with *H. pylori* infection have increased immunoglobulin G (IgG) antibody levels compared with subjects without *H. pylori* infection (5, 7, 13, 22), and IgG antibody levels decrease significantly after successful treatment of the infection (13). Most serological tests developed for diagnostic use have sensitivities and specificities of between 70 and 90% (8, 11, 16).

The serological cross-reactions between *H. pylori* and other bacteria that may cause false-positive results are still incompletely investigated (6, 12, 21). It has been possible to pinpoint several antigens that were specific for *H. pylori* by immunoblot analysis (3). Of these antigens, five bands had a relative molecular mass in the range of 15 to 30 kDa (3). The purpose of

this study was to purify these low-molecular-mass (LMW) antigens of *H. pylori* by simple ultrafiltration, to evaluate this antigen preparation in an enzyme-linked immunosorbent assay (ELISA) for routine detection of IgG antibodies to *H. pylori*, and to compare it with a previously used heat-stable (HS) antigen preparation in sera from consecutive children with abdominal symptoms and consecutive adult patients with dyspeptic symptoms.

MATERIALS AND METHODS

Patients and serum samples. Serum was obtained from (i) 76 consecutive Czech children and young adults (median age, 13 years; range, 5 to 20 years) with abdominal pain as their major symptom, admitted to hospital for endoscopy, and (ii) 151 consecutive adult Danish patients (median age, 52 years; range, 21 to 85) admitted to hospital for endoscopy because of dyspepsia. The majority of the adult patients were treated with H₂ blockers prior to endoscopy.

Endoscopy, histology, and growth of *H. pylori*. All patients had an upper endoscopy, and at least four biopsies were obtained from the antral part of the stomach during endoscopy. Tissue sections of formalin-fixed biopsies were stained with hematoxylin-eosin to evaluate the morphology. Additional silver staining was done to evaluate the presence of *Helicobacter*-like organisms (HLO). Biopsies from adults were examined by two pathologists, and biopsies from children were examined by a third pathologist. Biopsies from antral corresponding to biopsies taken for histology were cultured under microaerobic conditions on 7% lysed horse blood agar plates at 37°C for up to 6 days. *H. pylori* was identified according to conventional laboratory methods (23).

Bacterial strain. A clinical isolate of *H. pylori* (CH-20479) from an adult male with duodenal ulcer has previously been tested (3, 5) and was used for the antigen preparations.

Whole-cell preparations. The bacteria were cultured under microaerobic conditions on lysed horse blood agar plates for 24 to 48 h, harvested, and washed two times in phosphate-buffered saline (PBS, pH 7.4). The preparations were centrifuged at 7,000 × g for 10 min, and the pellet was stored at -20°C. The pellets were resuspended in PBS (pH 7.4) to a concentration of 0.5 g (wet weight) per ml of PBS when used.

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Sonicated cell preparations. Whole-cell preparations (0.5 g [wet weight] per ml of PBS) were broken by sonication at 20,000 Hz for 45 s, which was repeated five times with a RapiDiss 300 19-mm probe with a 9.5-mm tip. The preparations were cooled during sonication by immersion in ice water. The sonicated bacteria were centrifuged at 11,000 \times g for 30 min, and the supernatants were stored at -20°C.

LMW antigen preparation. The supernatants of the sonicated cell preparations were filtered through a filter with a pore size of 0.2 μ m, then through a filter with a cutoff at 100,000 kDa (Millipore catalog no. UFP2THK24), and finally through a filter with a cutoff at 30,000 kDa (Millipore catalog no. UFP2THK24) mounted on a syringe. The filtered preparations were dialyzed against PBS (pH 7.4) in a dialysis bag with a cutoff at 12,000 kDa for 2 days with one exchange of PBS.

HS antigen preparation. The preparation of the HS antigen has been described previously (22). The bacteria were boiled for 2 h in PBS (pH 7.2) containing 1% Triton X-100, sonicated five times for 45 s each with 60-s intervals, and centrifuged at 11,000 \times g for 30 min.

SDS-PAGE. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method has previously been described in detail (3). The whole-cell preparation, the ultrasonicated cell preparation, and the filtered preparations were diluted 1:30 in PBS (pH 7.4) and mixed with equal volumes of sample buffer containing 0.4% SDS and 4.8% (w/vol) α -dithiothreitol. The suspensions were boiled for 5 min in a waterbath. Electrophoresis was carried out as described by Laemmli (14) with a 15% separation gel and a 5% stacking gel. Relative molecular weight kit: Pharmacia Fine Chemical). Coomassie brilliant blue staining was performed as described by Weber and Osborn (28). Silver staining was performed with silver nitrate after fixation with 10% glutaraldehyde and developed with 75 μ l of 24.5% formalin in 100 ml of 3% sodium carbonate.

Immunoblot analysis. Immunoblot analysis was done by a modification of the technique described by Towbin et al. (24) carried out as described previously (3).

Indirect ELISA. The ELISA method for HS antigen preparation has been described previously (5, 27). The same method was adapted for the LMW antigen preparation (protein concentration, 0.53 g/liter). The microtiter plates were coated overnight at room temperature with the LMW antigen preparation diluted 1:100. The antigen coating was optimized by checkerboard titration. Serum samples from adults were diluted 1:800 and those from children were diluted 1:100 because of the much higher IgG antibody levels to *H. pylori* in adults than in children. The assays were done in triplicate with an uncoated well as a control for nonspecific binding of IgG or conjugated anti-human IgG to the plastic matrix. After incubation for 1 h at room temperature, the plates were washed, and horseradish peroxidase-conjugated rabbit antibodies to human serum IgG antibodies were added to each well. The plates were incubated for 1 h at room temperature and washed, and enzyme activity was detected by using the ortho-phenylenediamine dihydrochloride- H_2O_2 system. The chromogenic reaction was stopped with H_2SO_4 after 15 to 30 min, and the optical density (OD) was read in a photometer at 492 nm. The antibody amount was expressed as ELISA units (EU), which are OD values corrected for day-to-day and plate-to-plate variation. The predictive values, sensitivities, and specificities were calculated by standard methods.

RESULTS

LMW antigen preparation. Figure 1 demonstrates the SDS-PAGE pattern of the LMW antigen preparation compared with the sonicated preparation and the preparation after filtration through different filters and dialysis. It can be seen that several antigens with molecular masses above 30 kDa and below 15 kDa are still present in the LMW antigen preparation, but in clearly reduced amounts compared with those antigens with molecular masses of between 15 and 30 kDa.

***H. pylori* status.** All patients were considered *H. pylori* positive if either *H. pylori* was cultured or HLO were seen in tissue sections of the biopsies. Forty (53%) of 76 children and 83 (55%) of 151 adults were found to be *H. pylori* positive (Table 1).

IgG antibody response to *H. pylori* in children. The distributions of the antibody levels to both HS and LMW antigen according to *H. pylori* status are shown in Table 1. The best IgG antibody discrimination between *H. pylori*-positive and *H. pylori*-negative subjects was found when the cutoff for negative results was <100 EU and the cutoff for positive results was \geq 200 EU, with a gray area between 100 and 199 EU for the LMW antigen (Table 1). With these cutoff levels, none of 36 *H. pylori*-negative children were seropositive and only 1 (2.8%) of them was borderline seropositive. Samples from 2 (5%) of 40 *H. pylori*-positive children did not respond to the LMW antigen, and 3 (7.5%) were borderline seropositive (Table 1). By

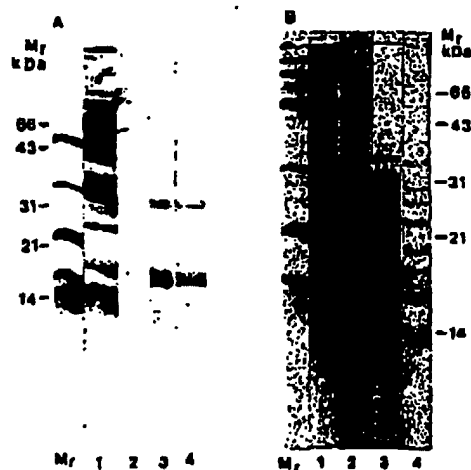


FIG. 1. Coomassie stained (A) and silver-stained (B) SDS-PAGE of whole-cell sonicate of *H. pylori* (lane 1), fraction below 100 kDa (lane 2), and fraction below 30 kDa, before (lane 3) and after (lane 4) dialysis through a membrane with a cutoff at 12 kDa. M_r , relative molecular mass.

using these cutoff levels for detection of antibodies to LMW antigens in children, the sensitivity, specificity, predictive value for a positive result (PVP), and predictive value for a negative result (PVN) were 95, 100, 100, and 95%, respectively. The best distribution for the HS antigen was obtained when the gray zone was set between 400 and 799 EU. The sensitivity, specificity, PVP, and PVN were 82, 80, 85, and 77%, respectively, for the HS antigen.

IgG antibody response to *H. pylori* in adults. By using identical cutoff levels for adults and for children, the sensitivity, specificity, PVP, and PVN were 99, 45, 72, and 96% for the LMW antigen and 96, 36, 70, and 85% for the HS antigen, respectively. By changing the lower cutoff level to 200 and the upper cutoff level to 599 for adults for the LMW antigen, the sensitivity, specificity, PVP, and PVN were optimized to 95, 73, 80, and 93%, respectively. By changing the upper cutoff level to 799 and keeping an unchanged lower cutoff level for adults for the HS antigen, the sensitivity, specificity, PVP, and PVN were optimized to 96, 35, 70, and 85%, respectively. Both the PVPs and the specificities seem to be rather low, because of a great proportion of seropositive patients among the *H. pylori*-negative patients (Table 1).

Analysis of the patients according to *H. pylori* status. Of all *H. pylori*- or HLO-positive adult patients, 94% were detected by culture and only 6% were detected by microscopy of histological sections alone.

Analysis of the false-positive adult patients. All 21 adult patients were admitted for dyspeptic symptoms, and 9 (43%) had histological chronic gastritis. Four (19%) had peptic ulcer, four (19%) had endoscopic gastritis, and two (9.5%) had gastric cancer. Only five (24%) patients had normal endoscopic findings and normal morphology of the stomach biopsies. Two of these five patients had very high antibody levels to *H. pylori* LMW and HS antigens. Antibodies to *H. pylori*-specific antigens were confirmed in all 21 seropositive patients by Western blot analysis.

DISCUSSION

A high prevalence of antibodies against five LMW antigens in the range from 15 to 30 kDa (bands H to L) in patients with

TABLE 1. IgG antibody response to *H. pylori* LMW and HS antigens in children with abdominal pain and adults with dyspeptic symptoms

ELISA result (EU)	<i>H. pylori</i> status results (no. of patients)							
	Children				Adults			
	LMW antigen		HS antigen		LMW antigen		HS antigen	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Neg
>1,000	14	0	24	3	41	9	63	2
800-1,000	2	0	4	2	5	3	8	1
600-800	3	0	2	6	10	2	6	1
400-600	6	0	4	5	11	7	3	1
200-400	10	0	1	9	13	10	3	1
100-200	3	1	3	5	2	12	0	1
<100	2	35	2	6	1	25	0	1
Total	40	36	40	36	83	68	83	68

* *H. pylori* infection was confirmed by culture and/or microscopy.

H. pylori infection compared with subjects without *H. pylori* infection was demonstrated in a previous study (3). In this study, a filtration method was used to purify these antigens. The protein bands with molecular masses greater than 40 kDa were diminished compared with the five low-molecular-mass bands, as shown in Fig. 1. Minor amounts of a broad range of proteins were, however, still present in the antigen preparation. It is suggested that these additional proteins are of less importance than the predominant low-molecular-mass protein fraction for the detection of the antibody response to *H. pylori*, even though small amounts of antigens which cross-react with other bacteria may be present in the preparation (3).

This antigen preparation was found to be very reliable for detection of *H. pylori* infections in children by ELISA, with predictive values, sensitivities, and specificities of 95 to 100% and with a rather narrow gray zone of 100 to 199 EU. This is a great improvement over the HS antigen and may be an advantage over other antigen preparations used for the serological detection of *H. pylori* infections in children (28, 29). *H. pylori* or HLO were detected by both microscopy of histological sections and culture in all children, which is in accord with other studies (25), whereas *H. pylori* or HLO were detected in 6% of the adult patients by microscopy alone. The PVP and the specificity were rather low for adult dyspeptic patients compared with children, even with an extended gray zone of between 100 and 399 EU (Table 1). The results obtained with ELISA, however, were better for adults with the LMW antigen than with the HS antigen. The low PVPs and specificities were due to 21 seropositive patients with negative culture and without HLO by microscopy. Thirteen (62%) had very high antibody levels to *H. pylori* LMW antigen, and 16 (76%) of the 21 patients had either morphological or endoscopic abnormalities in the stomach. IgG antibodies to *H. pylori*-specific antigens were confirmed in all 21 patients by Western immunoblotting.

It is impossible to establish whether the disagreement between culture/microscopy and antibody detection is caused by an underestimation of the *H. pylori* infection by culture/microscopy or caused by low antibody levels in patients with an eradicated or suppressed *H. pylori* infection. It is therefore difficult to establish a satisfactory gold standard. This is in agreement with a previous study in which the adult population was used to evaluate commercial kits, and low PVPs and specificities of the test results were found (10). An important reason for these low values was probably the transportation time from the clinic to the laboratory, which might diminish the number of *H. pylori*, as a previous study of another population with a shorter transportation time showed higher PVPs of the

test results (11). Another reason for this might be the number of biopsies taken from each patient, which was only half the number recommended in the Sydney classification (20). Alternatively, a majority of these patients could have had an *H. pylori* infection previously, even though four of these patients had peptic ulcer, which occurs 16% of the total number of patients with peptic ulcer included in this study, and in addition three (12%) patients with peptic ulcer were borderline seropositive but *H. pylori* infection negative. This is an unusually high number of *H. pylori*-negative patients with peptic ulcer compared with that found in other studies. This study reflects the difficulties in using consecutive unselected patients for evaluation of serological tests for detection of infections with fastidious microorganisms.

The *H. pylori* LMW antigen has improved IgG antibody detection as a predictive factor for *H. pylori* infection over use of the HS antigen and other first-generation tests. Detection of IgG antibodies to LMW antigens appears to be reliable for detection of *H. pylori* infection in children, who are difficult to endoscopy, whereas use of the LMW antigen seems to be of similar benefit as in most other commercial kits for adults.

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